# Regulation of Elongation Factor-2 Kinase by pH†

Maxim V. Dorovkov, Karen S. Pavur, Alexey N. Petrov, and Alexey G. Ryazanov*

*Department of Pharmacology, University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854*

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ABSTRACT: Elongation factor-2 kinase (eEF-2K) is a $Ca^{2+}$/calmodulin-dependent protein kinase that phosphorylates and inactivates eEF-2 and that can regulate the rate of protein synthesis at the elongation stage. Here we report that a slight decrease in pH, within the range observed in vivo, leads to a dramatic activation of eEF-2K. The activity of eEF-2K in mouse liver extracts, as well as the activity of purified recombinant human eEF-2K, is low at pH 7.2−7.4 and is increased by severalfold when the pH drops to 6.6−6.8. eEF-2K requires calmodulin for activity at neutral as well as acidic pH. Kinetic studies demonstrate that the pH does not affect the $K_M$ for ATP or eEF-2 and activation of eEF-2K at acidic pH is due to an increase in $V_{max}$. To analyze the potential role of eEF-2K in regulating protein synthesis by pH, we constructed a mouse fibroblast cell line that expresses eEF-2K in a tetracycline-regulated manner. Overexpression of eEF-2K led to a decreased rate of protein synthesis at acidic pH, but not at neutral pH. Our results suggest that pH-dependent activation of eEF-2K may play a role in the global inhibition of protein synthesis during tissue acidosis, which accompanies such processes as hypoxia and ischemia.

Eukaryotic elongation factor-2 (eEF-2) is a 100 kD protein that catalyzes the translocation of peptidyl-tRNA from the A to the P site on the ribosome, resulting in movement of the ribosome along mRNA during the elongation phase of translation [reviewed in ref *1*]. eEF-2 kinase (eEF-2K) is a highly specific protein kinase that phosphorylates eEF-2 [reviewed in refs *2* and *3*]. Phosphorylation of eEF-2 by eEF-2K results in eEF-2 inactivation and a concomitant inhibition of protein synthesis (*4*). Thus, the proposed function of eEF-2K is to regulate protein synthesis rate at the elongation stage.

eEF-2K was cloned and sequenced (*5*, *6*) and appeared to be a member of a new class of protein kinases, named α-kinases, which have no sequence similarity to the conventional eukaryotic protein kinases (*6*−*8*). The other members of the α-kinase class are myosin heavy chain kinase A and B from *Dictyostelium* (*9, 10*) and several other protein kinases from mammals, including two unusual bifunctional proteins, in which an α-kinase domain is covalently linked to an ion channel belonging to the TRP superfamily: channel kinase 1 (ChaK1, TRPM7) and channel kinase 2 (ChaK2, TRPM6) (7, *11*−*14*).

eEF-2K is a $Ca^{2+}$/calmodulin-dependent enzyme; therefore, it is also known as $Ca^{2+}$/calmodulin-dependent protein kinase III (*15*). It is a highly specific kinase: the only known substrate it phosphorylates is eEF-2 [reviewed in ref *2*]. eEF-2 phosphorylation is observed in all vertebrate tissues tested, and in tissue extracts, eEF-2 is one of the most prominently phosphorylated proteins [reviewed in ref *2*]. Several regulators of eEF-2K have been found that can provide a clue to the physiological function of eEF-2K. Since its activity depends on $Ca^{2+}$, it was implied that eEF-2K could be regulated by $[Ca^{2+}_i]$. There are several reports showing transient eEF-2 phosphorylation in vivo when $[Ca^{2+}_i]$ is known to be elevated. For example, an increase in eEF-2 phosphorylation has been observed during the transition of cells from quiescence into proliferation (*16*) and during mitosis (*17*). It is not clear, however, if this transient eEF-2 phosphorylation can lead to inhibition of protein synthesis. For example, it was recently demonstrated that activation of L-type $Ca^{2+}$ channel activity in $GH_3$ pituitary cells, which results in an increase in intracellular $[Ca^{2+}_i]$, led to moderate phosphorylation of eEF-2 without an observed inhibition of translation (*18*).

It has also been found that eEF-2K can be regulated by cAMP through cAMP-dependent protein kinase (PKA). PKA phosphorylates eEF-2K, leading to a calcium-independent activity of the kinase in vitro (*19*). Furthermore, treatment of rat 3T3-L1 adipocytes or rat myeloid IPC-81 cells with agents that increase the intracellular level of cAMP (isoproterenol or CPT-cAMP) causes eEF-2K activation and inhibition of protein synthesis (*20*, *21*). Also, it has been demonstrated that stress-activated protein kinase SAPK4/p38δ (*22*), $p90^{RSK1}$ and p70 S6 kinase (*23*) can phosphorylate eEF-2K and regulate its activity. Therefore, there exists a network of signaling pathways regulating eEF-2K.

In this study we provide evidence that pH could be another physiological regulator of eEF-2K. The intracellular pH in animal cells and tissues is maintained near neutrality (7.2−7.4); however, there are many common normal and pathological situations when the intracellular pH changes. Such events include gamete activation and fertilization, progression of cells through the cell cycle, as well as various examples of stimulus−response coupling [reviewed in refs *24* and *25*]. Acidification of the cytoplasm (down to pH 6.4−6.8) also occurs in tissues during ischemia and hypoxia (*26*). Acidi-

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* To whom correspondence should be addressed. E-mail: ryazanag@umdnj.edu. Phone: (732) 235-5526. FAX: (732) 235-4073.

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fication leads to a global inhibition of protein synthesis [see, e.g., refs *27*−*30*], however, the mechanism by which protein synthesis is inhibited at acidic pH is unclear.

Here we report that the activity of eEF-2K undergoes dramatic changes within a narrow physiological pH range. Specifically, eEF-2K activity was low at a normal pH of 7.2−7.6 and was dramatically activated when the pH was reduced to 6.6−6.8. This pH-dependent activation of eEF-2K may play a role in a global protein synthesis inhibition observed during tissue acidosis, which accompanies such processes as hypoxia and ischemia.

## MATERIALS AND METHODS

*Materials.* Buffer reagents and other chemicals were obtained from Sigma. Radioisotopes were from DuPont/NEN. PCR reagents and restriction enzymes were from GIBCO/BRL and Roshe. Anti-eEF-2K antibody was from BD Transduction laboratories. Rabbit reticulocyte eEF-2 was purified as described (*31*). Expression and purification of recombinant human GST-eEF-2K was performed as described (*8*). Calmodulin was a kind gift of Dr. D. Wolff (Department Pharmacology, UMDNJ). Peptide MH-1 was synthesized commercially by Genosys and had the following sequence: RKKFGESEKTKTKEFL.

*eEF-2K Activity Assay with Purified eEF-2.* Recombinant GST-EF-2 kinase (1 $\mu$g) was incubated with purified rabbit reticulocyte eEF-2 (0.5 $\mu$g) in a reaction mixture consisting of 50 mM Hepes−KOH with variable pH, 10 mM magnesium acetate, 5 mM DTT, 100 $\mu$M $CaCl_2$, 0.5 $\mu$g calmodulin, 60 $\mu$M ATP, and 1 $\mu$Ci [$\gamma$-$^{33}$P]-ATP (3000 Ci/mmol) or 1 $\mu$Ci [$\gamma$-$^{32}$P]-ATP (3000 Ci/mmol). The total volume of the reaction was 40 $\mu$L. The reactions were run at 30 °C for 10 min, and were terminated by incubation in an ice/water bath and addition of Laemmli sample buffer. Samples were analyzed by 8% SDS−PAGE and autoradiography.

*Measurement of eEF-2 Phosphorylation in Mouse Liver Extract.* The tissue extract was produced similar to the S-10 preparation described by Morley and Jacson (*32*). In short, the extract was prepared in buffer solution containing 70 mM Hepes−KOH pH 7.2, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 10 mM $\beta$-mercaptoethanol. The homogenate was clarified by centrifugation for 10 min at 10 000*g* at 4 °C. Two $\mu$L of extract was used in eEF-2K activity assays as described above, but without addition of calmodulin and with or without addition of purified rabbit reticulocyte eEF-2.

*eEF-2K Activity Assay with Peptide Substrate.* Recombinant GST-eEF-2K (2 $\mu$g) was incubated with 100 $\mu$M MH-1 in a reaction mixture buffered with 50 mM Hepes−KOH (variable pH) unless mentioned otherwise (in one experiment 10 mM Hepes−KOH, MOPS−KOH, or imidazole were used), and containing 10 mM magnesium acetate, 5 mM DTT, 100 $\mu$M $CaCl_2$, 0.5 $\mu$g calmodulin, 100 $\mu$M ATP, and 1 $\mu$Ci [$\gamma$-$^{33}$P]-ATP (3000 Ci/mmol). The total volume of the reaction mixture was 50 $\mu$L. The reactions were run at 30 °C for 10 min and were terminated by incubation in an ice/water bath. An aliquot of each reaction was spotted onto a 2 cm × 2 cm square of phosphocellulose paper and then washed 5 times for 4 min in 100 mM phosphoric acid. After a 30 s rinse in 95% ethanol, the filter papers were dried and counted in a scintillation counter.

*Determination of Kinetic Parameters of eEF-2K.* Recombinant GST-eEF-2K was incubated with varying quantities of MH-1 peptide or purified eEF-2. The reaction mixture containing eEF-2 as a substrate consisted of 50 mM Hepes−KOH pH 6.6 or 7.4, 10 mM magnesium acetate, 5 mM DTT, 100 $\mu$M $CaCl_2$, 0.5 $\mu$g calmodulin, 1 mM ATP, 25 $\mu$Ci of [$\gamma$-$^{32}$P]-ATP (3000 Ci/mmol), and 0.3 $\mu$g of eEF-2K. The total volume of the reaction mixture was 20 $\mu$L. The reactions were run for 10 min at 30 °C and stopped by incubation in an ice/water bath and addition of Laemmli sample buffer. Samples were then analyzed by 8% SDS−PAGE and autoradiography. Bands corresponding to eEF-2 were excised from the gel and then quantitated by Cerenkov counting. The kinetic parameters of eEF-2K for ATP were determined using MH-1 peptide as a substrate. The reaction mixture consisted of 50 mM Hepes−KOH pH 6.6 or 7.4, 10 mM magnesium acetate, 5 mM DTT, 100 $\mu$M $CaCl_2$, 0.5 $\mu$g calmodulin, 100 $\mu$M of MH-1 peptide, 3 $\mu$g of eEF-2K, and varying concentrations of ATP and [$\gamma$-$^{32}$P]-ATP.

*Construction of a Cell Line with Regulated Overexpression of eEF-2K.* We used a Tet-off system to regulate the expression of eEF-2K in cells. The retroviral vector, pSIT, and cells expressing the Tc-responsive transcriptional activator (tTA) were kind gifts of Alexey Ivanov (Institute of Molecular Biology, Moscow). The vector was constructed [as described in (*33*)] on the basis of retroviral self-inactivating pSIH4 vector, which now is also available as pSIR (Clontech), by the insertion of a DNA fragment containing a minimal CMV promoter under the control of the tet-responsive element (TRE) derived from pUHD 10-3 (*34*). The cells were first established by 3T3 protocol from mouse embryonic fibroblasts, followed by stable introduction of the tTA gene into the cells (*35*).

The cell line with regulated expression of eEF-2K was constructed as follows. The pSIT retroviral vector bearing human eEF-2K cDNA under the control of the tetracycline responsive promoter was used to produce viruses in Retro-Pack packaging cell line (Clontech). The 3T3 cell line expressing tTA were infected with the produced viruses. Since the effectiveness of retroviral infection was very high (∼90%) and almost all cells survived $G_{418}$ selection, experiments were performed using mass cultures.

In the presence of tetracycline, the cells expressed only endogenous levels of eEF-2K. Removal of tetracycline from the medium led to overexpression of exogenous eEF-2K gene above endogenous levels of eEF-2K.

*Cell Culture and Measurement of Protein Synthesis In Cells.* 3T3 cells were maintained in DMEM (Sigma) with or without bicarbonate supplemented with 10% FCS (Hyclone), glutamine (Gibco/BRL), and penicillin/streptomycin (Gibco/BRL). Cells were grown in DMEM with bicarbonate in 7% $CO_2$ at 37 °C. When the pH of the medium needed to be adjusted, DMEM without bicarbonate was used. In this case the medium was supplemented with 50 mM Hepes. The pH of the complete medium was adjusted with 1 M KOH, and the medium was then filter-sterilized. Cell cultures with Hepes-buffered medium were maintained in an incubator with normal atmosphere (no additional $CO_2$) at 37 °C.

For experiments in which eEF-2K was overexpressed, 50 000 cells per well were plated in 48 well plates, with or without tetracycline. After cells reached confluence (in about 2 days) the medium was replaced with Hepes-buffered medium containing 20 $\mu$Ci/ml of [$^3$H]-leucine (172 Ci/mmol). After 24 h [$^3$H]-leucine was washed out and cells were

incubated for 0−3 h in Hepes-buffered medium containing 2 $\mu$Ci/ml of [$^{14}$C]-L-amino acid mixture. Cells were washed 3 times in ice-cold phosphate buffered saline (PBS), fixed with 50% methanol in PBS for 30 min, washed three times with 5% trichloroacetic acid and three times with methanol. Incorporated label was dissolved in 1 M NaOH overnight, neutralized with 1 M HCl, and counted in a scintillation counter using double $^{14}$C/$^{3}$H channels. The amount of $^{3}$H label reflects the total protein, and incorporated $^{14}$C reflects short-term changes in the rate of protein synthesis.

Measurement of protein synthesis in $GH_3$ pituitary cells was performed as described (*36*).

*Western Blotting with anti-eEF-2K Antibody.* Western blotting analysis using the anti-eEF-2K antibody was performed according to manufacture's instructions (BD Transduction laboratories). In short, cells were washed with ice-cold PBS on ice and dislodged by scraping with a rubber policeman. Laemmli sample buffer was added to lyse the cells, and the samples were boiled for 5 min. Samples were resolved on 8% SDS-acrylamide gel, transferred to PVDF membrane, stained using the mouse anti-eEF-2K antibody as primary and anti-mouse horseradish peroxidase conjugated antibody as secondary (Amersham). Specific eEF-2K staining was detected using ECL as a substrate (Cell Signaling) followed by exposure to X-ray film (BioRad).

*Measurement of Intracellular pH.* Intracellular pH was measured by loading BCECF/AM into $GH_3$ cells in balanced salt solution buffered at pH 7.4 for 30 min. One set of cells was washed and resuspended in normal balanced salt solution at 0.2 pH unit intervals between 6.0 and 7.8. A duplicate set of cells were resuspended in potassium buffer containing nigericin. A ratio of excitation at 485/440 with emission at 530 nm was determined for each sample, and $pH_i$ was calculated as described by Thomas et al. (*37*).

## RESULTS

*eEF-2 Phosphorylation in Liver Tissue Extracts.* Our studies of the effect of pH on eEF-2 phosphorylation was prompted by the report of Gillette et al. (*38*), who found that acidic pH selectively stimulated phosphorylation of a protein with properties similar to eEF-2 in mollusk neuron extracts. It had been shown previously that phosphorylated eEF-2 in liver extracts can be easily identified as a major phosphorylated band (*39*). Therefore, we analyzed the phosphorylation status of eEF-2 in mouse liver extract at varying pH values, ranging from pH 6.4 to 7.8. A strong increase in eEF-2 phosphorylation at acidic pH was observed (Figure 1A). Several phosphorylated proteins could be distinguished on the autoradiogram, and eEF-2 is the only protein whose phosphorylation was significantly increased as the pH was lowered. With the exception of a 80 kD band whose intensity was decreased, the intensity of other bands were not significantly affected by a decrease in pH. As expected, the phosphorylation of purified rabbit reticulocyte eEF-2 added to mouse liver extract was also strongly enhanced by a reduction in pH (Figure 1B). Thus, we found that phosphorylation of endogenous as well as exogenously added eEF-2 in mouse liver extract was strongly increased at acidic pH.

*Effect of pH on eEF-2 Phosphorylation by Recombinant eEF-2K.* We investigated whether the increase in eEF-2 phosphorylation was due to activation of eEF-2K. Purified reticulocyte eEF-2 was incubated with purified recombinant eEF-2K at varying pH values, ranging from 6.4 to 7.6. We found that both the autophosphorylation and phosphorylation activities of eEF-2K were strongly pH-dependent (Figure 2). The ability of the kinase to phosphorylate purified eEF-2 and to autophosphorylate was low at pH 7.2−7.6. However, eEF-2K activity was significantly increased when the pH of the reaction was lowered to 6.4−6.8.

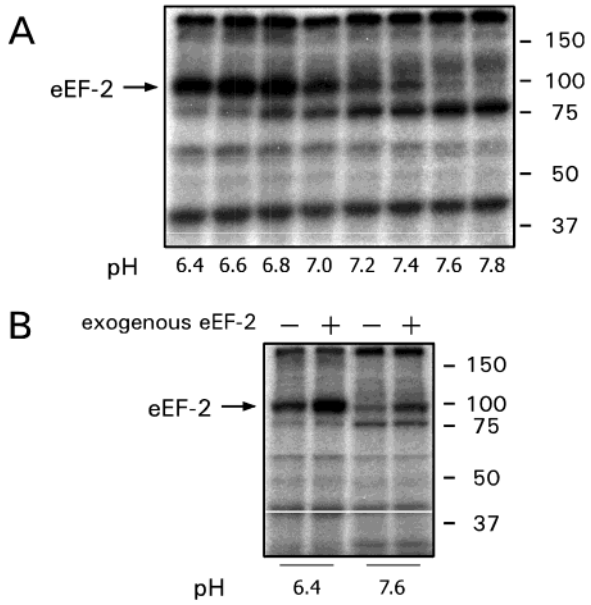


FIGURE 1: pH dependence of protein phosphorylation in mouse liver extract. The extract was added to a reaction mixture containing Hepes−KOH buffer at varying pH (see Materials and Methods). Samples were analyzed by 8% SDS−PAGE and autoradiography. Protein phosphorylations at different pH values: (A) in mouse liver extract and (B) in mouse liver extract with or without addition of purified rabbit reticulocyte eEF-2.

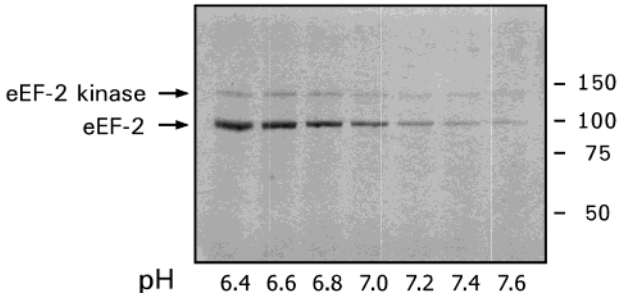


FIGURE 2: pH dependence of eEF-2K activity. Recombinant GST-tagged eEF-2K was incubated with purified rabbit reticulocyte eEF-2 and [$\gamma$-$^{33}$P]-ATP in a reaction mixture containing Hepes−KOH buffer with variable pH as described in Materials and Methods. Samples were analyzed by 8% SDS−PAGE and autoradiography.

*Effect of pH on Phosphorylation of Synthetic Peptide by Recombinant eEF-2K.* We had previously found that eEF-2K can efficiently phosphorylate a 16mer peptide (MH-1 peptide) corresponding to the site in *Dictyostelium* myosin heavy chain that is phosphorylated by myosin heavy chain kinase A, another member of the α-kinase class (*8*). This enabled us to use a simple biochemical assay to study eEF-2K activity without the possibility of contamination with other eukaryotic proteins.

As can be seen in Figure 3A, eEF-2K phosphorylated the peptide at a significantly higher rate at pH 6.6 compared to

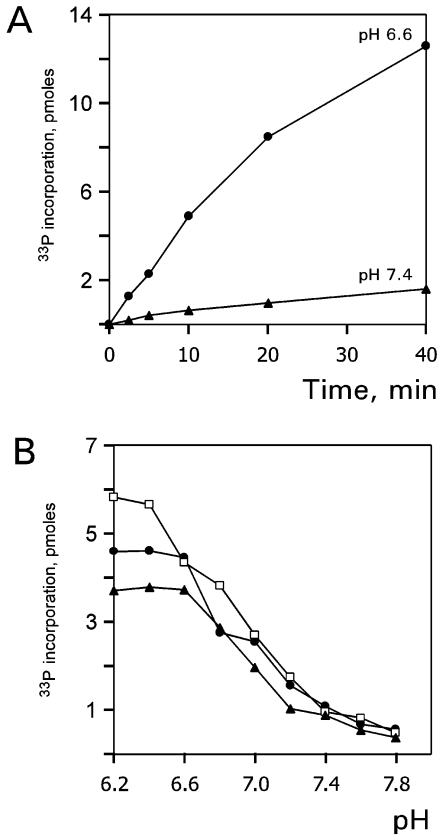


FIGURE 3: pH dependence of the synthetic peptide phosphorylation by eEF-2K. eEF-2K activity was assayed by phosphorylation of the peptide substrate MH-1 as described in Materials and Methods. Recombinant GST-tagged eEF-2K was incubated with the peptide in a reaction mixture containing different buffers with varying pH in the presence of [$\gamma$-$^{33}$P]-ATP. Reactions were spotted onto phosphocellulose paper, washed, and counted in a scintillation counter. (A) Time dependence of MH-1 phosphorylation by eEF-2K at pH 6.6 (circles) and 7.4 (triangles). (B) Phosphorylation of MH-1 by eEF-2K in three different buffers: 10 mM Hepes−KOH (circles), 10 mM MOPS−KOH (squares), and 10 mM imidazole−HCl (triangles). The concentration of buffers used in this experiment was 10 mM because we found that activity of eEF-2K was inhibited 2−3-fold in 50 mM imidazole.

pH 7.4. We further studied the pH dependence of the peptide phosphorylation in three different buffer systems (Hepes−KOH, MOPS−KOH, and imidazole−HCl). We used imidazole since it has been shown that $K^+$ and other monovalent cations are inhibitory for eEF-2K (*40*), and in contrast to Hepes and MOPS, imidazole is not titrated with KOH. As shown in Figure 3B, the pH dependence of eEF-2K in different buffers was similar. The eEF-2K activity in phosphorylating the peptide was severalfold higher at pH 6.4−6.6 than at pH 7.2−7.4.

*Kinetic Analysis of eEF-2K at Different pH.* The kinetic parameters of eEF-2K are shown in Table 1. Experiments were performed at both pH 6.6 and 7.4 with varying concentrations of eEF-2 or ATP. Kinetic parameters for eEF-2 or for ATP were determined using purified reticulocyte eEF-2 or using synthetic peptide MH-1, respectively. Kinetic analysis of eEF-2K at pH 6.6 and pH 7.4 demonstrated that pH had no significant effect on the $K_M$ for ATP or the $K_M$ for eEF-2. Changes in the magnitude of kinase activity at the different pH values tested were primarily due to changes in $V_{max}$, which was about 10-fold higher at pH 6.6 than at pH 7.4.

Table 1: Determination of Kinetic Parameters of eEF-2K at Different pH[a]

| | pH 6.6 | pH 7.4 |
|---|---|---|
| $K_M$ for ATP, $\mu$M | 200 | 210 |
| $K_M$ for eEF-2, $\mu$M | 1.82 | 2.2 |
| $V_{max}$ for eEF-2, nmol/min/mg | 4 | 0.4 |

[a] Experiments were performed as described in Materials and Methods. Experiments were done in Hepes−KOH reaction mixtures in the presence of [$\gamma$-$^{32}$P]-ATP at both pH 6.6 and 7.4 with different concentrations of purified eEF-2 or ATP. Kinetic parameters for eEF-2 or ATP were determined using purified reticulocyte eEF-2 or synthetic peptide MH-1, respectively.

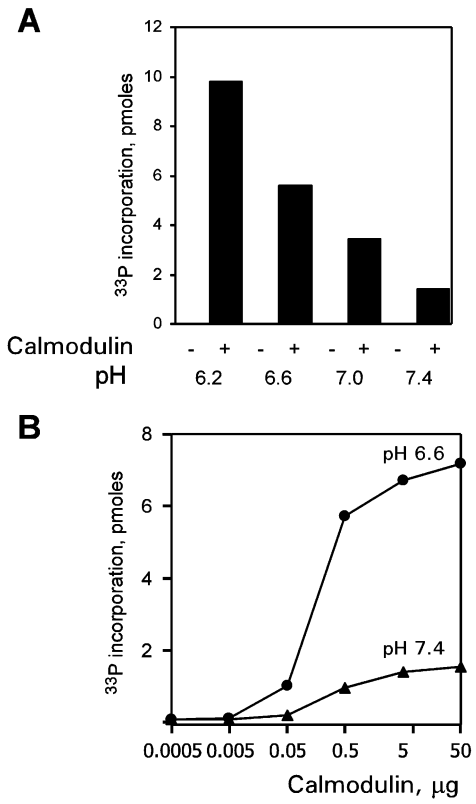


FIGURE 4: Calmodulin dependence of eEF-2K activity at different pH. eEF-2K activity was assayed by phosphorylation of the peptide substrate MH-1 as described in Materials and Methods. Recombinant GST-tagged eEF-2K was incubated with the peptide in a reaction mixture with varying pH in the presence of [$\gamma$-$^{33}$P]-ATP. Reactions were spotted onto phosphocellulose paper, washed, and counted in a scintillation counter. (A) MH-1 phosphorylation by eEF-2K at different pH values with or without calmodulin. (B) Calmodulin dependence of MH-1 phosphorylation by eEF-2K at pH 6.6 (circles) and 7.4 (triangles).

*Calmodulin Dependence of eEF-2K Activity at Different pH values.* It was suggested previously (*41*) that eEF-2K becomes calmodulin-independent at acidic pH values. However, we found that recombinant GST-eEF-2K was completely inactive without the addition of calmodulin at any of the pH tested, ranging from 6.2 to 7.4 (Figure 4 A). The activity of eEF-2K was dependent on calmodulin concentration at either pH 6.6 and 7.4 (Figure 4 B). Since experiments in the previous report were conducted with components purified from animal tissues, trace contaminating amounts of calmodulin could account for this discrepancy. For example, because the activity of eEF-2K is greatly increased at acidic pH, trace amount of copurified calmodulin could

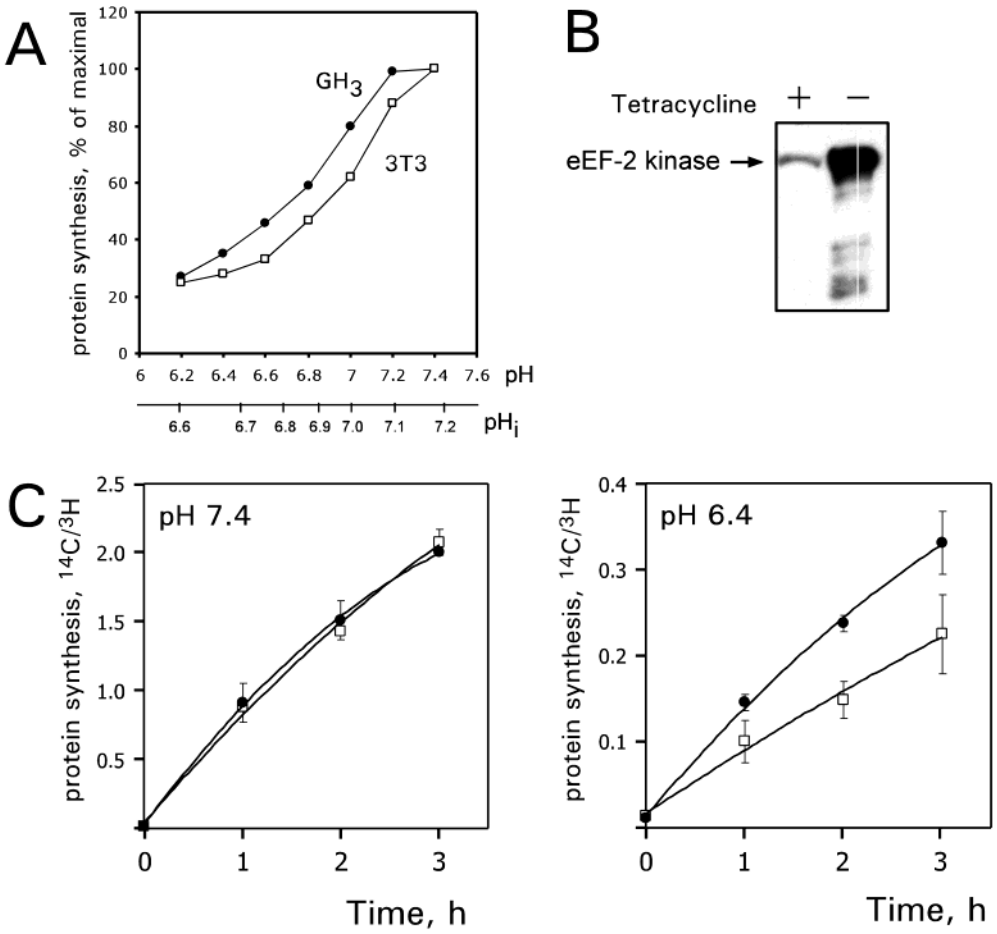


FIGURE 5: Effect of overexpression of eEF-2K on protein synthesis in mouse fibroblasts at different pH. (A) Dependence of protein synthesis rate on extracellular and intracellular pH in NIH 3T3 and $GH_3$ cells. (B) Western blotting of lysates prepared from Tet-off cells (with regulatable eEF-2K expression) incubated with or without tetracycline; the membrane was stained with anti-eEF-2K Antibody (see Materials and Methods). (C) The data for cells with normal levels (circles) or overexpression of eEF-2K (squares). Confluent cells were incubated with $^3$H-Leucine for 24 h at pH 7.4 and then with $^{14}$C-amino acid mixture in medium with pH 6.6 or 7.4 for different periods of time. For the time of the whole experiment, cells were continuously maintained with or without tetracycline. Protein synthesis was measured in cells at different pH as described in Materials and Methods. Samples were analyzed by scintillation counting in double $^{14}$C /$^3$H channels. The number of counts for $^{14}$C label was normalized to the amount of $^3$H.

be sufficient to elevate eEF-2K activity at acidic pH but not at neutral pH.

*Effect of eEF-2K Overexpression on Protein Synthesis in Mouse Fibroblasts.* It is well documented that a decrease in pH leads to a global inhibition of protein synthesis [see, e.g., refs *27*−*30*]. A decrease in extracellular pH from 7.4 to 6.2 caused a decrease in intracellular pH from 7.2 to 6.6, resulting in a significant inhibition of protein synthesis both in $GH_3$ and NIH 3T3 cells (Figure 5A). Since significant activation of eEF-2K occurred within the same pH range from 7.2 to 6.6, the observed inhibition of protein synthesis at acidic pH could be attributed in part to the activation of eEF-2K and the resultant phosphorylation of eEF-2. To investigate whether eEF-2K directly plays a role in the inhibition of translation by acidic pH in vivo, we constructed a cell line in which the expression of eEF-2K could be regulated. In this system (Tet-off) the overexpression of eEF-2K is induced by removal of tetracycline from the medium (see Materials and Methods). To construct the cell line, mouse 3T3 cells expressing the Tc-responsive transcriptional activator (tTA) were infected with pSIT retroviral vector bearing eEF-2K cDNA under the control of the tetracycline responsive promoter. In the presence of tetracycline, the cells expressed only endogenous levels of eEF-2K. However, when the medium was replaced with tetracycline-free medium, the exogenous eEF-2K gene was also expressed, leading to a dramatic overexpression of eEF-2K. Figure 5B shows the expression of eEF-2K in the constructed cell line after 2 days in medium with or without tetracycline.

The constructed cell line was used to examine whether the overexpression of eEF-2K has an effect on protein synthesis. Since eEF-2K becomes active at acidic pH, we expected that overexpression of eEF-2K would lead to stronger inhibition of protein synthesis at the lowered pH. Indeed, overexpression of eEF-2K did result in an increased inhibition of protein synthesis when cells were incubated in medium at pH 6.4 (Figure 5C). However, when cells were incubated in medium at pH 7.4, overexpression of eEF-2K did not have any effect on protein synthesis rate (Figure 5C).

## DISCUSSION

Here we report that eEF-2K is dramatically activated by a slight decrease in pH, within the pH range that is observed in vivo. The phosphorylation of eEF-2 in tissue extracts, purified eEF-2, as well as a synthetic peptide by purified recombinant eEF-2K were strongly pH dependent. To the

best of our knowledge, this is the first demonstration of a protein kinase that is strongly and directly activated by acidic pH within a physiologically relevant pH range. Our study may provide a link connecting acidification, observed during hypoxia or ischemia, with global inhibition of protein synthesis. It is well-known that hypoxia and ischemia result in acidosis that is accompanied by the inhibition of protein synthesis. This inhibition of protein synthesis plays an important role in preserving cellular ATP during hypoxia and ischemia [reviewed in refs *25*, *26*, and *42*].

We investigated the possible mechanisms for an unusual pH dependence of eEF-2K and analyzed the kinetics of eEF-2 phosphorylation at neutral and acidic pH values. Since the $K_M$s for ATP and eEF-2 did not change with pH, the activation of eEF-2K by acidic pH could not be attributed to an increased affinity of eEF-2K for eEF-2 or ATP. The results indicate that the increase in eEF-2K activity at acidic pH was primarily due to an increased $V_{max}$. The increase in autophosphorylation of eEF-2K as well as in phosphorylation of a synthetic peptide at acidic pH indicate that this activation was due to intrinsic properties of eEF-2K itself and not due to pH dependent changes in eEF-2.

The pH dependence of eEF-2K could be a result of the protonation/deprotonation of certain amino acid residues with p$K_a$s in the pH range 6.4−7.4. For example, the calmodulin-binding region of eEF-2K contains three histidines, which could be protonated/deprotonated within the pH range under study. Interestingly, these histidines are located at the positions that are usually occupied by positively charged residues in other calmodulin-dependent proteins. The positively charged residues in calmodulin-binding domains are not essential for the high-affinity binding of calmodulin but, rather, play an important role in the activation of calmodulin-binding enzymes after binding of calmodulin [reviewed in ref *43*]. Therefore, it can be speculated that the presence of these histidines in the calmodulin-binding domain of eEF-2K may play a role in the pH-dependent activation of eEF-2K. These histidines could be protonated at acidic pH, providing for a proper interaction with calmodulin and resulting in high levels of kinase activity.

There are many studies that report that acidification leads to a global inhibition of protein synthesis in vivo [e.g., *27*−*30*]. We also found that protein synthesis in mouse NIH 3T3 and $GH_3$ pituitary cells was inhibited approximately 4-fold when $pH_i$ was decreased from 7.2 to 6.6 (see Figure 5 A). This decrease in protein synthesis correlated with the activation of eEF-2K in vitro within the same pH range. The experiments with regulatable expression of eEF-2K in cells showed that overexpression of eEF-2K led to increased inhibition of protein synthesis at pH 6.4 but not at pH 7.4. This indicates that eEF-2K can play a role in the inhibition of protein synthesis by acidic pH in vivo. Although eEF-2K possesses measurable activity at pH 7.2−7.4, overexpression of eEF-2K did not in itself affect protein synthesis rate in cells at this pH. The most likely explanation for this is that, if the rate-limiting stage in the overall translation at pH 7.4 is initiation rather than elongation, then the decrease in the elongation rate, up to certain degree, will not have a significant effect on the overall translation rate. The other possibility is that the activity of eEF-2K in the cell is limited by certain factors, such as availability of calmodulin, kinases that phosphorylate eEF-2K, etc., and therefore eEF-2K activity in vivo may not be directly proportional to the amount of expressed eEF-2K protein. Overall, the fact that overexpression of eEF-2K leads to inhibition of protein synthesis only at acidic pH underscores the importance of pH in regulation of eEF-2K.

There are several other reactions in protein synthesis that could be regulated by pH in the physiological range of pH 6.4−7.4. For example, translation in a rabbit reticulocyte cell-free system is inhibited at acidic pH, and the observed effect is much stronger for capped mRNAs compared to that for uncapped mRNAs, indicating that interaction of initiation factors with the cap may be pH-dependent (*44*). It has also shown that acidic pH stimulates binding of elongation factor-1α (eEF-1α) to F-actin. This could sequester eEF-1α and prevent it from interacting with aminoacyl-tRNAs and ribosomes (*45*, *46*). The activity of ribosomal peptidyl-transferase is also strongly pH-dependent (*47*−*50*). Therefore, it is likely that a decrease in intracellular pH leads to the coordinated inhibition of protein synthesis at several stages of translation and that pH-dependent phosphorylation of eEF-2 is responsible for the inhibition of ribosomal translocation. It is reasonable to suppose that in order to stop the complex translational machinery, the coordinated inhibition of translation at several separate stages is required. Such coordinated inhibition of several reactions involved in protein synthesis by acidic pH, where activation of eEF-2K inhibits the traslocation phase of translation, could be the mechanism of global translational arrest that is observed during hypoxia and ischemia.

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