Stimulation-induced degradation of eukaryotic elongation factor-2 kinase (EF2K) by the ubiquitin-proteasome system

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Introduction

•Increases and decreases in neuronal activity can bidirectionally remodel the protein composition of the synapse, which involves both *de novo* protein translation and ubiquitin-proteasome system (UPS)-mediated protein degradation.[1]
•Both protein synthesis and degradation are required for late-phase long-term potentiation (L-LTP), and the balance or coordination of these processes appears important, as simultaneous inhibition of both leaves L-LTP intact.[2]
•***The mechanisms by which protein synthesis and UPS-mediated protein degradation are modulated by activity, and how they may directly balance or synergize, remain to be elucidated.***

•Eukaryotic elongation factor-2 kinase (EF2K), formerly termed CaMKIII, is a Ca^{2+}/calmodulin-dependent protein kinase that inhibits the elongation phase of translation by phosphorylating and inactivating eukaryotic elongation factor-2 (eEF-2)[3].
•While increased eEF-2 phosphorylation leads to an overall decrease in protein synthesis, it can paradoxically increase translation of certain transcripts, such as α-CaMKII or Arc[5,6], and conversely loss of EF2K is sufficient to abolish the rapid translation of Arc or MAP1B in response to mGluR stimulation, and also abolishes mGluR-mediated long-term depression.[7,8]
•***Together these data suggest that modulation of EF2K activity can remodel the translational activity of neurons, or sub-cellular compartments such as dendrites and synapses, serving perhaps as a signal amplification mechanism that shifts translation from house-keeping functions to those involved in stabilizing long-term plasticity.***

•In PC12 cells, prolonged stimulation with NGF, IGF-1, or forskolin decreases EF2K activity and protein level, suggesting a mechanism by which extracellular stimuli may globally facilitate translation.[9]
•***We hypothesized that the observed stimulation-induced reduction of EF2K results from its phosphorylation-dependent targeting to the UPS, a mechanism that could directly link activity-induced UPS function to altered patterns of protein synthesis, which in turn may support long-term forms of plasticity.***
•Consistent with this idea, Ser-440 and Ser-444 of EF2K reside in a consensus sequence (DSGYPS) for the phospho-degron motif recognized by the F-box proteins βTRCP-1 and βTRCP-2, which serve as the substrate receptors for the $SCF^{\beta TRCP}$ type of E3 ubiquitin ligase[10] and previous studies in glioblastoma cells have suggested that EF2K is a UPS target.[11]
•***Here, we show that EF2K is turned over in a proteasome-dependent manner in response to stimulation in PC12 cells and cortical neurons, that activation of PKA is sufficient to decrease EF2K levels in a manner similar to phospho-mimetic mutation of Ser-499, and that βTRCP binds to and drives the poly-ubiquitination of EF2K.***

Methods

Cell line culture: PC12 cells were seeded onto 6- or 12-well plates coated with collagen-I (Sigma) and grown in RPMI-1640, supplemented with 10% horse serum and 5% FBS (all from Invitrogen). HEK 293-T cells were grown on un-coated plates in DMEM (Invitrogen), supplemented with 10% FBS.

Stimulation of PC12 cells: Cells were transferred to serum-free media 1 hr prior to the addition of any reagents, and MG132 (Tocris, 25μM in DMSO) was added 30 minutes prior to the start of stimulation. Cells were stimulated with either forskolin (Tocris, 10 μM in DMSO) IGF-1 (Chemicon, 100 ng/mL in water), or 6-BNZ-cAMP (Sigma, 100μM in water) for 6 hr and lysed in buffer containing 1% NP-40, 50mM Tris, 200mM NaCl, and 1mM EDTA. For protein turnover experiments, cells were treated with cycloheximide (Sigma, 30 μg/mL in DMSO) in serum-containing media and lysed at the indicated times.

Primary cortical cultures: Primary cultures of rat cortical neurons (prepared at E18) were maintained in Neurobasal/B27 medium for 14-15 days in poly-L-lysine-coated plates. Forskolin (10μM) and BDNF (Chemicon, 100 ng/mL in water) were added to the conditioned media for 3 or 6 hr and cells were lysed as above. MG132 (25μM) was added 30 minutes prior to the start of stimulation.

Plasmids and Transfection: GST-F-box and FLAG-DN-Cul1 constructs were generous gifts from J.W. Harper. GFP-EF2K mutants were generated using standard procedures and validated by DNA sequencing. For transfections, DNA was incubated with Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) and added to cells. Protein expression was assayed at least 24 hr later.

Identification of phosphorylation sites by mass spectroscopy: EF2K was immunoprecipitated from HEK 293-T or PC12 cells and eluates were run on SDS-PAGE. EF2K bands were excised from stained gels and submitted to the W.M. Keck facility for TiO_2 phosphopeptide enrichment and LCMS.

GST pull-downs: HEK cells were transfected with FLAG-EF2K and GST-tagged F-box constructs. For each pull-down, 2 mg of protein was used, diluted in 1mL lysis buffer, and 50μL of glutathione sepharose beads (50% slurry, GE Healthcare) was added for 30 minutes at 4°C. The beads were washed three times in lysis buffer and bound proteins were eluted by boiling in 100 μL 5X sample buffer.

EF2K ubiquitination experiments: PC12 cells were transfected with GFP-EF2K, with or without GST-βTRCP-1 and treated with MG132 (25μM) for 2 hr to accumulate poly-ubiquitinated proteins. Lysates were boiled in 1% SDS for 10 min, which was diluted to 0.1% prior to the addition of monoclonal GFP antibodies overnight. Protein G beads (50% slurry, GE Healthcare) were added for 1 hr. The beads were washed three times and bound proteins were eluted by boiling in 1X sample buffer.

Western blotting and antibodies: Western blots were performed using standard techniques, imaged with a Licor Odyssey Infrared Scanner, and analyzed using Licor Odyssey software. Membranes were probed with Msα-GST (1:1000, Cell Signaling), Rbα-EF2K (Angus Nairn, 1:1000-500), Msα-GAPDH (Advanced Immuno Chemicals, 1:10000), and Ms αFLAG (Sigma, 1:1000).

Results

Figure 1: Stimulation of PC12 cells or cortical neurons causes proteasome-dependent degradation of EF2K.

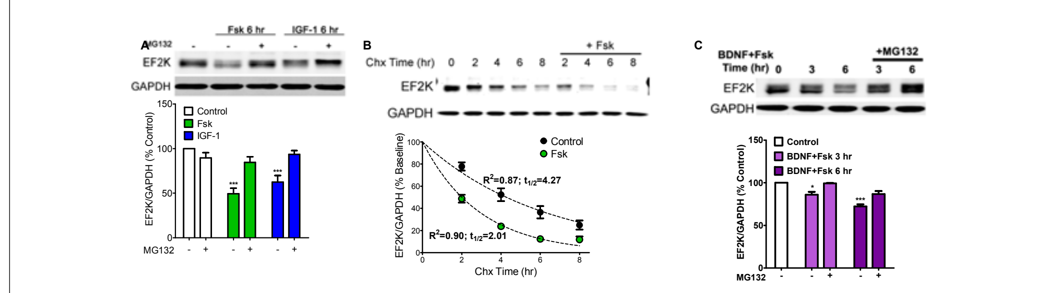
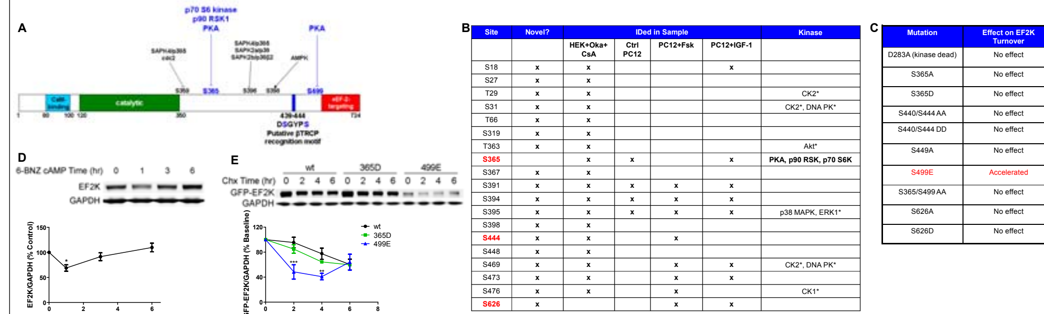


Figure 2: EF2K stability is regulated by phosphorylation.



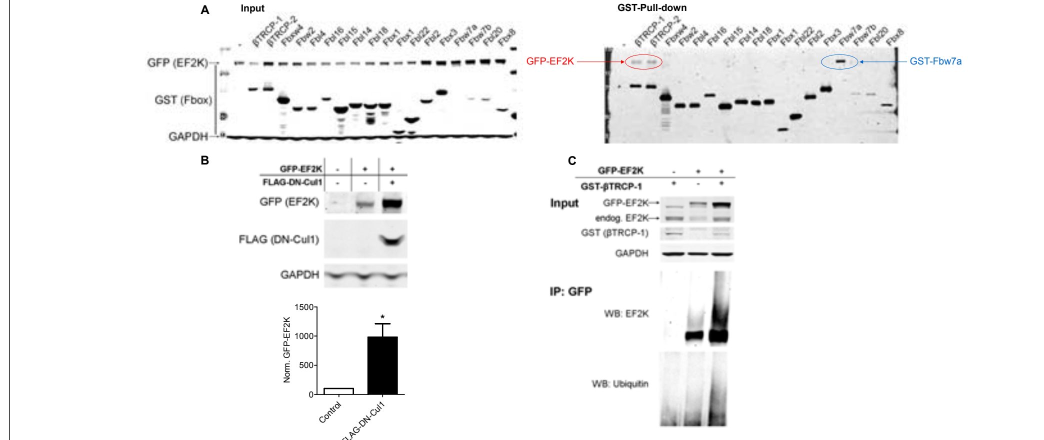
B

Site	Novel?	IDed in Sample				Kinase
		HEK+Oka+CsA	Ctrl PC12	PC12+Fsk	PC12+IGF-1	
S18	x	x			x	
S27	x	x				
T29	x	x				CK2*
S31	x	x				CK2*, DNA PK*
T66	x	x				
S319	x	x				
T363	x	x				Akt*
S365		x	x		x	**PKA, p90 RSK, p70 S6K**
S367	x	x				
S391	x	x	x	x	x	
S394	x	x	x	x	x	
S395	x	x	x	x	x	p38 MAPK, ERK1*
S398	x	x				
S444	x	x		x		
S448	x	x				
S469	x	x		x	x	CK2*, DNA PK*
S473	x	x		x	x	
S476	x	x		x		CK1*
S626	x			x	x	

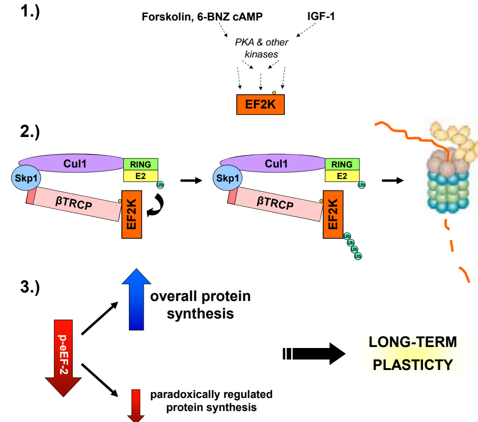
C

Mutation	Effect on EF2K Turnover
D283A (kinase dead)	No effect
S365A	No effect
S365D	No effect
S440/S444 AA	No effect
S440/S444 DD	No effect
S449A	No effect
S499E	Accelerated
S365/S499 AA	No effect
S626A	No effect
S626D	No effect

Figure 3: EF2K binds to βTRCP, EF2K stability is regulated by an SCF-type ubiquitin E3 ligase, and βTRCP drives poly-ubiquitination of EF2K.



Model



Conclusions

•Prolonged forskolin or IGF-1 stimulation in PC12 cells and combined forskolin and BDNF stimulation in cortical neurons reduces EF2K levels in a proteasome-dependent manner.

•Mass spectroscopy identifies many novel EF2K phosphorylation sites.

•Activation of PKA with 6-BNZ-cAMP reduces EF2K levels within 1 hr, which subsequently return to basal levels.

•Mutation of Ser-499 to Glu reduces EF2K steaty-state expression and accelerates EF2K turnover, with a subsequent return to low basal levels.

•EF2K binds to the F-box proteins β-TRCP-1 and β-TRCP-2.

•EF2K steady-state stability is enhanced by inhibiting Cul1 function.

•Over-expression of β-TRCP-1 increases EF2K poly-ubiquitination.

•***Phosphorylation-dependent UPS-mediated degradation of EF2K may represent a mechanism for coordination of protein degradation and translational control during long-term plasticity.***

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