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Original Paper

Nuclear ERK Translocation is Mediated by Protein Kinase CK2 and Accelerated by Autophosphorylation

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Key Words
ERK • MAPK • Nuclear Translocation • Importin7 • CK2

Abstract
Background/Aims: The extracellular signal-regulated kinases (ERK) 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family. Upon stimulation, these kinases translocate from the cytoplasm to the nucleus, where they induce physiological processes such as proliferation and differentiation. The mechanism of translocation of this kinase involves phosphorylation of two Ser residues within a nuclear translocation signal (NTS), which allows binding to importin7 and a subsequent penetration via nuclear pores. However, the regulation of this process and the protein kinases involved are not yet clear. ***Methods:*** To answer this point we developed specific anti phospho-SPS antibody, used this and other antibodies in Western blots and crystalized the phospho-mimetic mutated ERK. ***Results:*** Here we show that the phosphorylation of both Ser residues is mediated mainly by casein kinase 2 (CK2) and that active ERK may assist in the phosphorylation of the N-terminal Ser. We also demonstrate that the phosphorylation is dependent on the release of ERK from cytoplasmic anchoring proteins. Crystal structure of the phosphomimetic ERK revealed that the NTS phosphorylation creates an acidic patch in ERK. Our model is that in resting cells ERK is bound to cytoplasmic anchors, which prevent its NTS phosphorylation. Upon stimulation, phosphorylation of the ERK TEY domain releases ERK and allows phosphorylation of its NTS by CK2 and active ERK to generate a negatively charged patch in ERK, binding to importin 7 and nuclear translocation. ***Conclusion:*** These results provide an important role of CK2 in regulating nuclear ERK activities.

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Introduction

Extracellular signal-regulated kinase 1/2 (ERK) are central signaling proteins that mediate a variety of vital cellular processes including proliferation, survival, and even apoptosis [1-5]. In order to execute their functions, ERK molecules activate a large number of regulatory proteins, which are localized either in the cytoplasm or within various organelles including mainly the nucleus [6]. Indeed, the number of nuclear targets and downstream effectors of ERK, including a variety of transcription factors [7] is well over 200 [8]. These targets participate in the regulation of transcription as well as chromatin remodeling, and therefore play a central role in mediating most stimulated cellular processes [9]. Moreover, being such central signaling processes, dysregulation of these ERK-induced nuclear activities often leads to severe pathological processes including oncogenic transformation, neurodegenerative diseases, and developmental diseases [10]. In order to transmit their nuclear signals, ERK molecules that are localized in the cytoplasm of quiescent cells, rapidly translocate into the nucleus upon stimulation [11, 12]. Although many details on ERK in the nucleus were already provided, the mechanisms of their translocation are not fully worked out yet.

The nucleus is separated from the cytoplasm by a double membrane envelope [13]. Nuclear shuttling of proteins occurs through specialized nuclear pore complex (NPC), which ensure high selectivity of molecules for nuclear import/export supporting proper cytoplasmatic/nuclear molecular balance. The majority of proteins enter the nucleus by active transport mechanism, based on nuclear localization signal (NLS) in the shuttling proteins that binds to importin-α (Impα) and importin-β (Impβ) that act as shuttling transport factors through the NPCs [14]. However, a significant number of signaling proteins, which rapidly translocate into the nucleus upon cell stimulation, do not contain an NLS, and therefore must utilize distinct mechanisms for their translocation. In a previous study our group have identified a novel NLS-independent mechanism of stimulated nuclear translocation of signaling proteins including ERK, SMAD3 and MEK1 [15-17]. This mechanism involves a novel nuclear translocation signal (NTS), which contains either Ser or Thr residues that are phosphorylated upon stimulation to allow binding to importin7, and thereby nuclear shuttling via the NPCs. The NTS of ERK contains the sequence Ser-Pro-Ser (SPS) within its kinase insertion domain, which undergoes stimuli-dependent phosphorylation by as yet unknown kinases.

Protein Kinase CK2 (CK2), formerly known as casein kinase II, is a ubiquitous protein Ser/Thr kinase that plays a central role in the regulation of variety of cellular processes [18-20]. This kinase acts a tetramer containing two catalytic (α and/or α') and two regulatory (β) subunits [21]. It is a constitutively active kinase whose minimal consensus phosphorylation site is usually Ser-Xaa-Xaa-Glu/Asp, but additional Glu or Asp residues in some of the surrounding residues often allows the phosphorylation of more than 300 substrates identified [22, 23]. Upon phosphorylation, these substrates regulate variety of processes, including proliferation, transformation, apoptosis, senescence, and also malignant cell transformation [24, 25]. In the present paper we show that CK2 is the main kinase that phosphorylates both Ser residues within the ERK-NTS. The phosphorylation of one of the N-terminal Ser is facilitated by active ERK trans-autophosphorylation. We also demonstrate that the phosphorylation is dependent on ERK-release from hindering cytoplasmic anchors. Crystal structure showed that CK2 phosphorylation creates an acidic patch important for the translocation. These results provide an important role of CK2 in regulating ERK activity in the nucleus as well as proliferation and oncogenic transformation.

Materials and Methods

Reagents and Antibodies

Tetradecanoyl phorbol acetate (TPA), Epidermal Growth Factor (EGF), The CK2 inhibitors: 4, 5,6, 7 tetrabromobenzotriazole (TBB), and 2-dimethylamino-4, 5,6, 7-tetrabromo-1H-benzimidazole (DMAT), as well as TGF-4',6'-diamino-2-phenylindole (DAPI) were obtained from Sigma (St Louis, MI). Chelating sepharose-coupled nickel-nitrilotriacetic acid agarose beads and Resource-15Q anion exchange column were from Amersham (England). Secondary antibody (Ab) conjugates were from Jackson ImmunoResearch (West Grove, PA). Anti Histone H1 (sc-56403), tubulin (sc-23949), hemagglutinin (HA, sc-7392), anti-Elk-1 (sc-355), anti-RSK-1 (sc-231), anti-CK2α (sc-6479), and anti-MEK1 (sc-219) Abs were acquired from Santa Cruz Biotechnology (CA); anti-pElk-1 (2B1, Ser383, #9186) and anti-pRSK (Ser380, #9341) Abs were from Cell Signaling Technology (Beverly, MA); and anti green fluorescent protein (anti-GFP, 11814460001) Abs were from Roche Diagnostics GmbH (Mannheim, Germany). Anti-Imp7 Ab (H00010527-M07) was from Abnova (Taipei, Taiwan). Abs to doubly TEY-phosphorylated ERK (pTEY-ERK, M8159) and general ERK (gERK, M5670) were from Sigma (Rehovot, Israel), who also produced and purified the poly and monoclonal anti-phospho-SPS-ERK Abs.

DNA Constructs and Mutations

GFP-ERK2 was prepared in pEGFP-C1 vector (Clontech, Mountain View, CA). Human MEK1 was ligated into HindIII and ApaI sites downstream to red fluorescent protein (RFP) gene of DsRed1-N1 vector (Clontech), and GST-ERK2 constructs were prepared in pGEX vector. All mutations described were performed by site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. C-terminal tagged HA-CK2 wild type in pRc/CMV vector was a gift from Dr. Litchfield (University of Western Ontario, London, ON). 6His-MEK construct was produced as described [26]. All SiRNAs were from Dharmacon (Lafayette, CO).

Cell Culture and Transfection

HeLa, MDA-MB-231, MCF-7, and HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose, CHO cells were grown in F12-DMEM high glucose, all supplemented with 10% fetal bovine serum and 50 μM glutamine, but no pyruvate. Transfection was using either polyethylenimine, or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). SiRNAs were transfected using oligofectamin (Dharmacon). In order to reach equal transfection efficiencies transfected cells were divided to the necessary number of plates 24 h after transfection.

Immunofluorescence Microscopy

This was performed as described [27]. Cells were fixed in 3% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.2% Triton X-100 in PBS-BSA (2%) for 20 min at room temperature. The fixed cells were sequentially incubated with appropriated Abs, (diluted in PBS-BSA 1:200) for 1h, followed by either Cy-2 or rhodamine-conjugated secondary Abs and DAPI (diluted in PBS-BSA, 1:200) for 1 h. Slides were analyzed and photographed by a fluorescence microscope (600x magnification; Nikon, Japan).

ImageStream Analysis

Nuclear translocation of ERKs was examined by ImageStream system (Amnis Corp., Seattle, WA, USA) using the IDEAS image analysis program. About 1 million cells per sample were rinsed in cold PBS and gently scripted into 1.5ml tubes. Cells were spin down by centrifugation, fixed in 3% paraformaldehyde (20 min), and then permeabilized with 0.1% Triton X-100 in PBS-BSA (2%) (20 min, 23^0C). For endogenous ERK staining, fixed cells were sequentially incubated with anti-ERK Abs (1:200 in PBS-BSA; 1 h, 4^0C) following by incubation with Cy-2 conjugated secondary Abs (1:200) and propidium iodide (PI; 1μg/ml, for nuclear staining), in PBS contained 100μg/ml of RNase and 0.1 mM EDTA (1 h, 40C) The cells transfected with GFP-containing plasmids were incubated with secondary Abs and PI. The ERK1/2 green image and the nuclei red image were first compensated into separate channels, and then overlay of green and red colors (yellow color) was quantified. Additional information regarding to ImageStream technique can be found at the Internet site of the company (Amnis Corp), and ref [28].

Cell extraction, Cell Fractionation, Western Blotting, and Co-immunoprecepitation

(i) Preparation of cellular extracts for Western Blotting. This was performed as described [29]. Cells were rinsed twice with ice-cold PBS and scraped into Radio-immunoprecipitation assay buffer (RIPA; 137 mM NaCl, 20 mM Tris (pH 7.4), 10% glycerol, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, and 20 μM leupeptin). The extracts were centrifuged (20, 000x*g*, 15 min, 4°C), and the supernatants were further analyzed by Western boltting. The blots were developed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Abs.

(ii) Cellular fractionation. Cells were rinsed twice with ice-cold PBS, suspended in ice-cold hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2mM PMSF, 0.5mM DTT, and 0.5% Nonidet P-40), incubated (10 min, 4^0C), disrupted by repeated aspiration through a 20-gauge needle, and centrifuged (12,000 Ωxg, 5 min). The supernatant, containing the cytosolic fraction, was boiled in sample buffer. The pellet was suspended in extraction buffer (420 mM NaCl, 50 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 25% glycerol), incubated on ice for 30 min, sonicated (50 W, 2x 7 sec), and centrifuge (15,000xg, 30 min). The supernatant, containing the nuclear fraction, was subjected to Western blot with the appropriate Abs.

(iii) Co-immunoprecipitation. Cells were rinsed twice with ice-cold PBS and scraped into buffer H (50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin). The extracts were sonicated (50 W, 2x 7 sec), and centrifuged (15,000x*g*, 15 min, 4°C). Cellular extracts were incubated overnight at 4^0C with the appropriate Abs preconugated to A/G beads (1 h, 23^0C). Subsequently, the beads were washed x3 with coimmunoprecipitation washing buffer (20 mM HEPES pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 150 mM NaCl and 0.1% Triton) and once with PBS, and subjected to Western blot analysis.

Luciferase reporter assay

The following plasmids (from Forchheimer Plasmid Collection of the Weizmann Institute of Science, Rehovot Israel) were used for the experiment: FR-Luc (reporter plasmid), pFA2-Elk1 (fusion *trans*-activator plasmid), pFC2-dbd (negative control) and pFC-MEK1 (positive control). The plasmids were transfected into HEK-293T cells with Lipofect 2000 according to the manufacture protocol. Thirty-six h after transfection, cells were starved overnight, pretreated with TBB (10μM, 2 h) or left untreated as control and then stimulated with EGF (50ng/ml, 6 h) or left untreated as control. The assay was performed using Dual-Luciferase® Reporter Assay System (Promega, USA) in accordance to the manufacture protocol. The luminescence was detected using "Viktor^2 multi-label counter" (Perkin Elmer).

In-vitro kinase assay

Substrates used for in-vitro kinase assay: wild type or mutated GST-ERK2, 6His-MEK1 and dephosphorylated α-Casein (both from Sigma, St-Louis, MI). The substrates were mixed with cold ATP (0.5 mM), [γ^{32}P]-ATP (4000 cpm/pmole), and CK2 or active ERK2 (250 unit each, New England Biolabs, MA, USA); or GST-ERK2 (ERK), active GST-ERK2 (Act-ERK) or kinase dead GST-KA-ERK2 (KA-ERK) kinases produced in our lab, in an appropriate kinase buffers (supplied by the company; 1xCK2 buffer contained 20 mM Tris-Hcl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$; 1xERK2 buffer contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, o.o1% Brij35; both were done in a total volume of 20μl). The mixture was shacked at 900 rpm (30 min, 30°C), mixed with 4xSDS and boiled for 5 min. Proteins were separated by 12-15% SDS-PAGE, followed by Western blotting. The loading of α-Casein was assayed by protein staining with Reagent Blue (Pierce, IL, USA).

Expression and purification of ERK2 mutants for crystallization purposes

The SPE-ERK2 were expressed in *E.coli* strain Rosetta as N-terminally hexahistidine-tagged proteins. The bacteria were grown, lysed and then purified chelating sepharose coupled nickel - nitrilotriacetic acid agarose beads. Protein elution was obtained with a linear gradient of imidazol from 10 mM to 250 mM, using 50 mM Tris-HCl pH 8.0, 0.3 M NaCl and 250 mM imidazole buffer. The fraction that contained ERK2 protein was then dialyzed against 50mM Tris-HCl pH 8.0, 0.1M NaCl, 1mM EDTA for 4 hours at 4°C and then transferred to dialysis buffer containing Tris-HCl pH 8.0, 0.1M NaCl, 1mM DTT and 5% glycerol for 16-18 h. Protein solution was then subjected to a second purification step on a 23 ml Resource 15Q anion exchange column equilibrated with 50 mM Tris–HCl pH8.0, 0.1 M NaCl, 5% glycerol and 1 mM DTT. The protein was

eluted using a linear gradient of NaCl with the buffer as follows: 50 mM Tris–HCl pH8.0, 1 M NaCl, 5% glycerol and 1 mM DTT. Typical yield was 6 mg from one liter of bacteria. The purified protein was concentrated using Vivaspin (VivaScience) up to 6-12 mg/ml determined by absorption at 280 nm. The purified protein divided into aliquots and stored at –80°C.

Crystallization, data collection, and solution of the SPE-ERK2 mutant

The SPE-ERK2 were expressed in *E.coli* as described in the supplementary segment. Crystals of SPE-ERK2 protein were obtained by the vapor diffusion sitting drop technique by mixing equal amounts of protein solution (2.3-2.5 mg/ml) and reservoir solution in containing 61 mM Ammonium Sulfate, 100mM Bis Tris pH 7.0, 12-15% (v/v) PEG 3350. Streak seeding [30] using WT-ERK2 crystals was conducted in order to initiate crystallization. Crystal appeared within a day of incubation at 20ºC and reached the final size several days later. For data collection crystals were transferred into cryoprotectant solution containing the crystallization solution and 15% glycerol. Crystallographic data were collected at 100 K using an Oxford Cryostream cryosystem cooling device from a single crystal on an ADSC Quantum 315R CCD detector with an oscillation range of 1.0º at beam line ID23-1 at The European Synchrotron Radiation Facility (ESRF), Grenoble, France. The crystals belonged to the monoclinic space group $P2_1$ with one ERK2 molecule in the asymmetric unit (Table 1). Data were integrated, reduced and scaled using the HKL2000 suite [31]. The structures were solved via molecular replacement methods using Molrep [32] implemented in CCP4i suite [33] using the ERK2 structure (1ERK) as the search model after removing all solvent molecules. Following molecular replacement, the models were refined using rigid body and then restrained options REFMAC5 [34]. Solvent molecules were added utilizing ARP/WARP [35]. The models were fitted into electron-density maps using the graphics program Coot [36].

Table 1. Data collection and refinement statistics for $ERK2^{SPE}$ and $ERK2^{Q95A/EPS}$. [a]Rsym(I)=Σ|I-<I>|/ Σ I., [b]Test set consists of 5% for all data

	$ERK2^{SPE}$	$ERK2^{Q95A/EPS}$
ESRF beamline	ID23-1	ID14-4
Wavelength (Å)	0.98	0.93
Space Group	$P2_1$	H32
Unit Cell Parameters (Å)	a=48.8 b=70.0 c=60.1 β=109.0 º	a=87.6 b=87.6 c=307.6 γ=120.0 º
Resolution range (last resolution shell)	50.0-1.70 (1.73-1.70)	37.6-4.10 (4.17-4.10)
Unique Reflections	40797	3821
Redundancy	3.6	11.3
Rsym(I)[a] (%)	5.8 (70.3)	7.5 (64.4)
Completeness	97.1 (96.0)	100.0 (99.7)
I/σ	36.2 (1.7)	42.3 (4.1)
Number of protein atoms	2834	
Number of solvent atoms	161	
R-factor	18.8	
R-free[b]	24.5	
Average B factor (Å^2)		
Protein	36.2	
Solvent	40.8	
RMSD from ideality		
Bond Length (Å)	0.017	
Bond Angle (º)	1.7	
Ramachandran Plot (PROCHECK)		
Favored (%)	95.6	
Allowed (%)	4.1	
Generously Allowed (%)	0	
Disallowed (%)	0	

Results

CK2 is important for nuclear translocation of ERK

The two phosphorylated Ser residues of ERK's NTS lie within consensus phosphorylation sites of several protein kinases. Since the C-terminal Ser seems to fall within a CK2 site, we undertook to examine the role of the latter kinase in the nuclear translocation of ERK. For this purpose, we first pretreated CHO cells with CK2 inhibitors, stimulated the cells with TPA for 15 minutes, and followed the nuclear translocation of endogenous ERK by staining with an anti ERK antibody (Ab). As expected, most ERK molecules were localized in the cytoplasm of resting cell, and translocated into the nucleus after TPA stimulation (Fig. 1A). However, pretreatment with the selective CK2 inhibitors: TBB ([37], Fig. 1A) and DMAT ([38], data not shown) prevented the stimulated nuclear translocation, without any significant effect on the localization of ERK in resting cells. Similar effects of CK2 inhibitors were observed with MCF7 (Fig.1A), MBA-MB-231 and HeLa cells (data not shown). The effect of the CK2

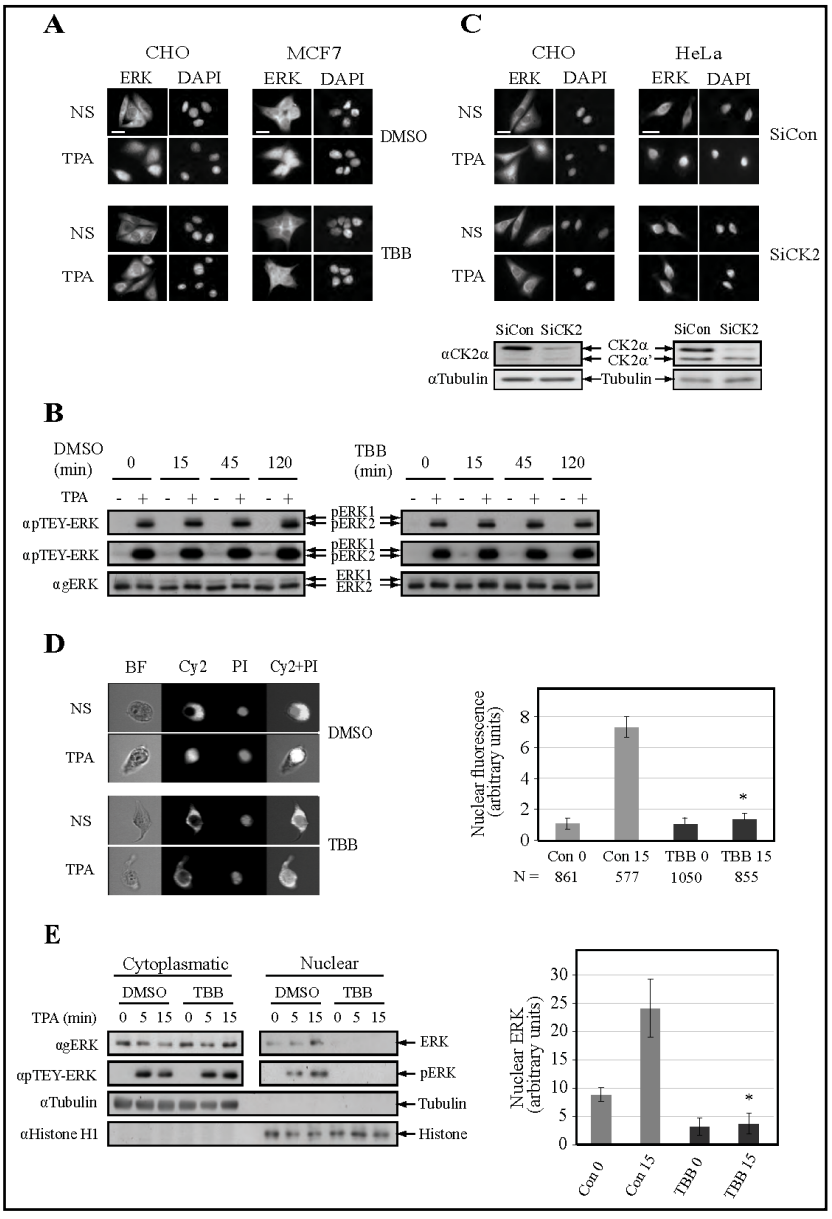

Fig. 1. CK2 plays a role in the nuclear translocation of ERK1/2. (A) CK2 inhibition prevents nuclear translocation of endogenous ERK1/2. CHO and MCF7 cells were serum-starved (16 h, 0.1%), pretreated with either TBB (10 μM, 2 h) or DMSO control, and then either stimulated with TPA (250nM, 15 min) or left untreated as control. The cells were stained with αgERK1/2 Ab and DAPI. Scale-bar – 15 μm. (B) TBB does not affect TEY-ERK phosphorylation. HeLa cells were serum starved, pretreated with TBB (10μM) or DMSO (indicated times) and then stimulated with TPA (250nM, 15 min). Cell extracts were subjected to Western blot analysis using αpERK (low exposure and high exposure) and αgERK Abs. (C) Knockdown of CK2 prevents nuclear translocation of endogenous ERKs. CHO and HeLa cells were transfected with either siCK2α or non-relevant siRNA (siCon). Thirty-six h after transfection the cells were serum starved, stimulated and processed as above. The efficiency of CK2α knockdown is shown as compared to endogenous tubulin levels. (D) Analysis of CK2 effect on the nuclear translocation of endogenous ERK1/2 by ImageStream. HeLa cells were serum and treated as above, and then the cells were analyzed by ImageStream. The graph represents fluorescence of nuclear ERK1/2 (two independent experiments), where the fluorescence of non-stimulated control was considered as 1. N - number of cells analyzed. * - P (student T-test) <0.02. (E) Cell fractionation confirms the CK2's role. HeLa cells were treated as in 1A. After fractionation, cytoplasmic and nuclear samples were subjected to Western blot using the indicated Abs. Tubulin and Histone H1 served as localization controls. The graph represents densitometric analysis of nuclear ERK1/2 (two independent experiments). * - P <0.05.

inhibitors was not due to modulation of the activatory TEY phosphorylation, as judged by Western blotting with anti pTEY Ab (Fig. 1B). Since pharmacological inhibitors are often not fully specific, we ascertained the CK2 effect by specifically knocking down its expression. For this purpose, we used siRNA of CSNK2A1 (α subunit of CK2), which reduced the expression

of CK2α (but not CK2α') in HeLa and (unexpectedly) CHO cells (Fig. 1C). As with the pharmacological inhibitors, also knock down of CK2α resulted in prevention of TPA-induced nuclear translocation of ERK (Fig. 1C).

In order to quantify our results, we used ImageStream Analyzer that simultaneously detects the localization of a fluorescent dye in many individual cells, thus obtaining statistically significant data. For this purpose, we treated HeLa cells with the CK2 inhibitor TBB, stimulated the cells with TPA for 15 min, and subjected them to the machine. Pictures of the sorted cells (see a representative cell in Fig. 1D left), as well as the quantification of the nuclear fluorescence of the sorted cells (Fig. 1D right), revealed that in great majority of resting cells the ERK molecules were localized in the cytoplasm. Upon stimulation, most ERK staining was shifted to the nucleus, while TBB pretreatment prevented the TPA-stimulated ERK translocation. Finally, another quantitative measure of nuclear translocation of ERK was the subcellular fractionation of the treated cells. Similar to the results in Fig 1D, this method also demonstrated that TBB significantly prevents nuclear accumulation of ERK upon stimulation (Fig. 1E). Taken together, our results strongly suggest that CK2 participates in the regulation of nuclear ERK translocation upon stimulation.

CK2 phosphorylates ERK's NTS and is involved in the activation of nuclear ERK targets

The finding that CK2 inhibitors prevent the nuclear translocation of ERK suggested that CK2 may act as an NTS kinase. In order to verify this point, we examined the ability of CK2 to phosphorylate purified GST-ERK2 *in vitro*. As expected, CK2 phosphorylated WT-ERK2, but not ERK2 mutated in the Ser residues within the NTS (ERK-APA; Fig. 2A); this indicates that the Ser residues within the NTS are the only CK2 sites within ERK2. This phosphorylation was abolished by TBB signifying that the phosphorylation is indeed mediated by CK2 and not caused by autophosphorylation. We then examined the *in vivo* effect of CK2 inhibition or knockdown on NTS phosphorylation using anti pSPS Ab, which primarily recognizes the doubly-phosphorylated NTS. We found that TBB as well SiCK2 from two different sources (Fig. 2B, C and data not shown) result in the prevention of NTS phosphorylation in HeLa and CHO cell lines. Since this effect is not dependent on changes in TEY phosphorylation, at any time after stimulation (Fig. 1B and 2C), it is likely that CK2 is not involved in the upstream activation of the ERK cascade, but rather directly phosphorylates the Ser residues in the NTS. We also found that MEK inhibition had a similar effect to that of CK2 inhibition, and a possible reason for it will be explained below. In order to further verify the role of the CK2 effect, we postulated that the impaired nuclear translocation of active ERK should lead to a decrease in the phosphorylation of nuclear ERK targets, without much change in the phosphorylation of cytoplasmic targets. Indeed, pretreatment of cells with CK2 inhibitor, or knockdown of CK2α resulted in a significant decrease in the phosphorylation and activation of the transcription factor Elk1, which is a well-known nuclear target of ERK (Fig. 2A,B, D). On the other hand, the phosphorylation of a cytoplasmatic ERK target (RSK) was reduced by MEK inhibition, but not significantly affected by the inhibition of CK2 activity. We also examined the effect of CK2 inhibition on cell viability, which is known to be regulated by the ERK cascade, and found that TBB indeed inhibits it in both non-transfected as well as cells overexpressing constitutively active MEK1. Interestingly, overexpression of the ERK2-EPE construct rescued the TBB effect (data not shown), indicating that although CK2 affects cell proliferation by inhibiting various cellular processes, its effect in our system might be mediated, at least in part, by the nuclear activity of ERK. These results clearly indicate that the NTS phosphorylation is executed primarily by CK2, acting downstream of the TEY phosphorylation, and therefore does not affect the activation of ERK. Thus, CK2 only affects the nuclear targets of ERK, without much influence on the cytoplasmic activity.

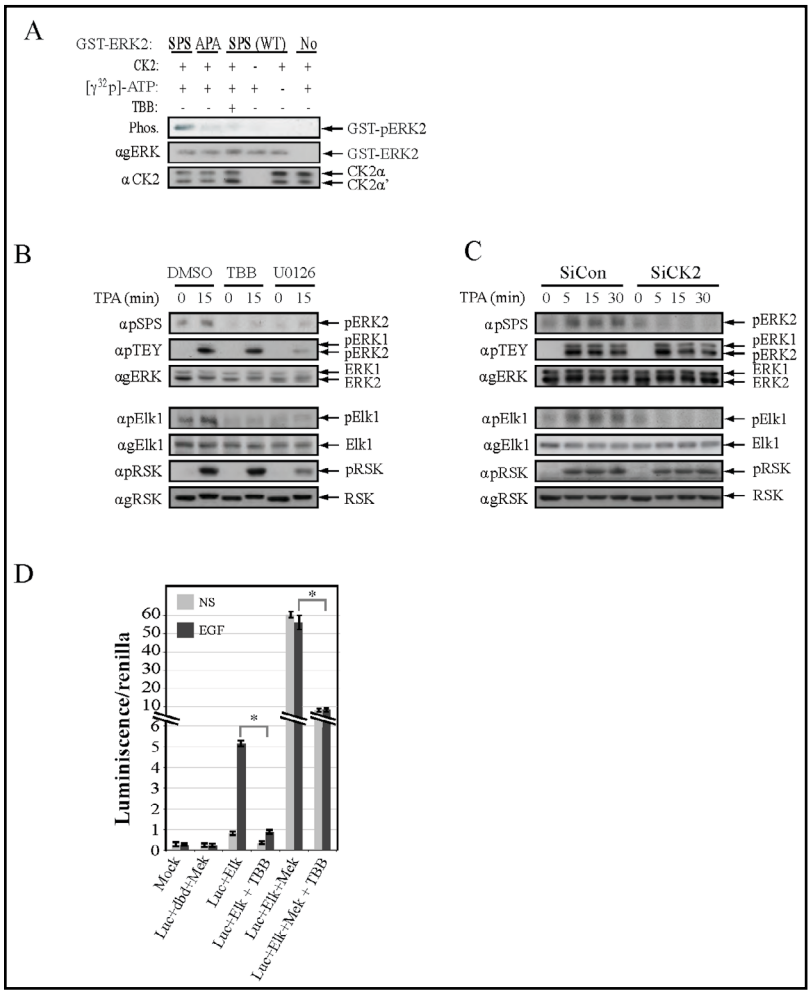

Fig. 2. CK2 phosphorylates ERK's NTS and is involved in the activation of nuclear ERK targets. (A) CK2 phosphorylates ERK's NTS in-vitro. The indicated GST-ERK2 constructs (0.1 μg per sample) were subjected to in-vitro CK2 phosphorylation, that was detected by autoradiography (upper panels). The loaded ERK and CK2 constructs were detected using αgERK and αCK2α Abs (middle and lower panels respectively). (B) CK2 inhibition prevents ERK's NTS and Elk1 but not RSK1 phosphorylation. HeLa cells were serum starved (0.1 %, 16 h), pretreated with TBB (10 μM, 2 h), UO126 (5μM; 2 h) or DMSO control, and then stimulated with TPA (250 nM, 15 min) or left untreated as control. Cell extracts were subjected for western blot analysis with the indicated Abs. (C) CK2 knockdown prevents ERK's NTS and Elk1 but not RSK1 phosphorylation. CHO cells were transfected with either siCK2 or non-relevant siRNA (siCon). Thirty six h after transfection, the cells were serum-starved and then either stimulated with TPA (250 nM, 15 min) or left untreated as a control. Cell extracts were subjected to Western blot analysis using the same Abs as in Fig. 2B. Efficiency of CK2 knockdown is shown in Fig.1B. (D) Inhibition of CK2 attenuates the ERK1/2-dependent activation of Elk1. HEK293T cells were transfected with the plasmids: pFC2-Elk1, constitutively active MEK1 (pFC-MEK1), and pFC2-dbd as control. The cells were then either pretreated with TBB (10μM, 2 h), or left untreated as control followed by stimulated with EGF (50 ng/ml) for 6 h where indicated. The graph represents the ratio of the luminescence of pFR-Luc (reporter)/renilla luminescence. The bar-graph is the mean and standard error of three repeats. * P<0.01.

The most important requirement for substrate recognition by CK2 within its consensus site, is the presence of acidic amino acid 3 amino acids C-terminal to the phosphorylated site (position +3), whereby acidic amino acid at position +2 may contribute to the recognition as well [39, 40]. In order to further verify the involvement of CK2 in NTS phosphorylation, we constructed GFP-conjugated mutants of ERK2, in which the Glu and Asp residues at positions of 248 and 249 (+2 and +3 from the phosphorylated Ser246) were changed to Ala, producing the sequence SPSQAA (ERK-SQAA). The same mutation was also inserted within an NTS phosphomimetic mutant of ERK2, producing the sequence EPEQAA (ERK-EQAA). These constructs were cotransfected into CHO cells together with red fluorescent protein (RFP)-conjugated MEK1. All mutants were localized in the cytoplasm of quiescent cells, indicating that they still preserve their ability to interact with MEK1. As previously

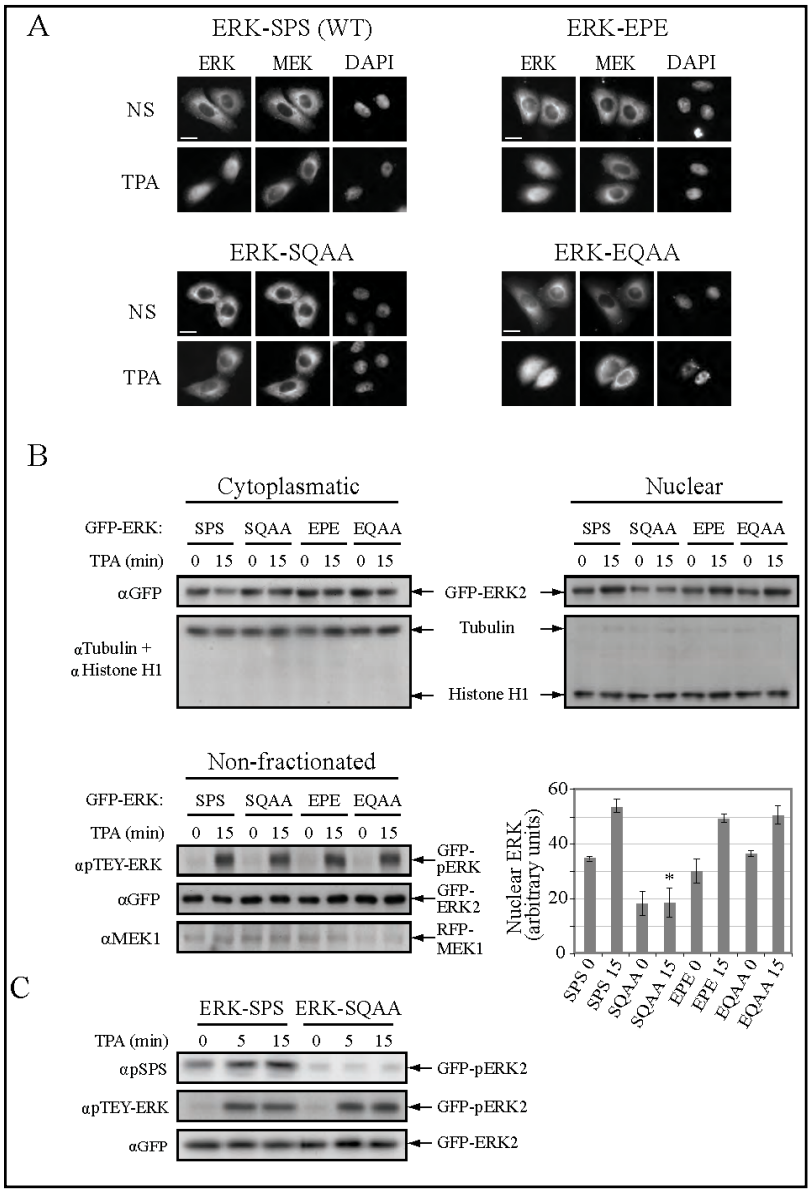

Fig. 3. Mutation of E248 and D249 prevents ERK's NTS phosphorylation and nuclear translocation. (A) Mutation of D248 and E249 inhibits nuclear translocation of exogenous ERK2. CHO cells were co-transfected with the following GFP tagged plasmids: ERK2-WT (SPSQED), ERK2-EPEQED, ERK2-SPSQAA (SQAA), or ERK2-EPEQAA (EQAA) and RFP-MEK1. Thirty-six h after transfection the cells were serum starved and either stimulated with TPA (250nM, 15 min) or left untreated (NS). The cells were stained by DAPI and visualized by using fluorescent microscope. Scale bar – 15 μm. (B) Cells were treated as describe in A, and then fractionated to cytosolic/nuclear fractions and subjected to Western blot analysis. Tubulin and histoneH1 serve as purity and loading markers. P-ERK as well as ERK and MEK expression were also detected using whole cell extracts (lower blots). The bar-graph represents densitometric analysis of nuclear ERK (three experiments). * P<0.05. (C) Mutation of D248 and E249 inhibits ERK's NTS phosphorylation. 293T cells were transfected with the indicated GFP tagged plasmids, serum-starved and stimulated as indicated. Cell extracts were subjected for Western blot analysis with the indicated Abs.

shown [15], TPA stimulation of the SPS (WT)-ERK- and EPE-ERK-expressing cells resulted in nuclear accumulation of ERK2 (Fig. 3A). TPA stimulation of the ERK-SQAA-expressing cells did not result in the nuclear translocation of the mutant, but the phosphomimetic mutant (ERK-EQAA) rescued the effect. Fractionation confirmed these results by showing that despite a lack of effect on TEY phosphorylation, ERK-EQAA is readily present in the nucleus, while SQAA is not (Fig. 3B). The abrogated nuclear translocation was not due to changes in TEY phosphorylation, as the NTS mutations had only minor effects on the activating phosphorylation (Fig. 3B, lower blot). Finally, Western blot analysis using anti pSPS Ab showed no TPA-induced phosphorylation of the Ser residues within the NTS (Fig. 3C). These results show the importance of the acidic 248/249 residues in the process.

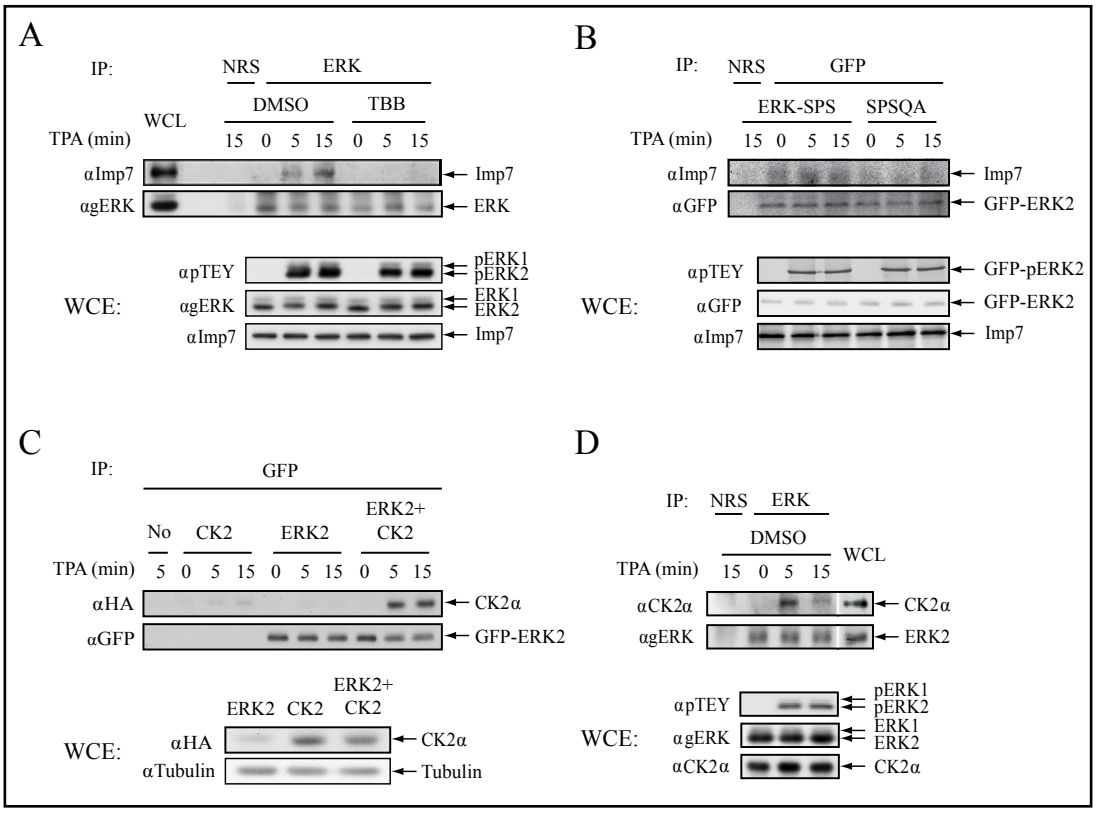

Fig. 4. ERK binding to Imp7 and CK2. (A) Imp7 interaction with ERK dependents on CK2 phosphorylation of ERK's NTS. HeLa cells were serum-starved and treated with TPA (250 nM) for the indicated times. ERK was immunoprecipitated using αgERK Abs or normal rabbit serum (NRS) that served as an irrelevant Ab control. Co-immunoprecipitated Imp7 was detected by Western blot analysis using αImp7 Abs (upper panel). ERK activation and Imp7 amount were determined using the indicated Abs. (B) Mutation of E248 and D249 prevents the interaction of ERK with Imp7. HEK-293T cells were transfected with the GFP tagged plasmids of ERK2-WT (SPS) and ERK2-SPSQAA, serum starved and stimulated with TPA (250 nM) for the indicated times. ERK constructs were immunoprecipitated using either αGFP Ab or NRS control, and co-immunoprecipitated Imp7 was detected by Western blot analysis with αImp Abs. ERK activation and Imp7 amount were detected using the indicated Abs. (C) Ectopically expressed CK2 interacts with overexpressed ERK2 in a stimulus-dependent manner. HEK-293T cells were transfected with HA-tagged CK2α, GFP-ERK2 or both plasmids. Thirty-six h after transfection, the cells were serum-starved and then stimulated with TPA (250 nM) for the indicated times. ERK2 was immunoprecipitated using αGFP Ab, and the co-immunopreciopitated CK2α was detected by Western blot analysis using αHA Ab. The amount of expressed proteins was detected with αHA and αTubulin Abs. (D) Stimulus-dependent interaction of endogenous CK2 and ERK. HeLa cells were serum-starved and then stimulated with TPA (250 nM) for the indicated times. Endogenous ERKs were immunoprecipitated using either αgERK Ab or NRS control, and co-immunoprecipitated CK2α was detected by Western blot analysis with αCK2α Ab. ERK activation and CK2 loading were detected using the indicated Abs.

ERK/Imp7 interaction is regulated by CK2 binding and phosphorylation of ERK

We have previously shown that the translocation of ERK to the nucleus is executed by the interaction of their phosphorylated NTS with Imp7 [15]. Since Imp7 recognizes only NTS-phosphorylated ERK, we examined whether CK2 inhibition affects the endogenous ERK/Imp7 interaction in stimulated cells. As expected, stimulation of HeLa cells with TPA resulted in an enhanced interaction of ERK with Imp7 (Fig. 4A). Inhibition of CK2 phosphorylation by TBB as well as prevention of NTS phosphorylation due to SQAA mutation prevented this interaction (Fig. 4A, B). These results clearly support the involvement of CK2-mediated NTS

phosphorylation in the interaction with Imp7 and thereby in the nuclear translocation of ERK. We then entertained the possibility that the phosphorylation of NTS by CK2 is dependent on its physical association with ERK. For this purpose, we overexpressed GFP-ERK2 and HA-CK2 in HEK-293T cells, which were starved, and then stimulated with TPA. The extracts of these cells were then used for coimmunoprecipitation with anti GFP Ab, which revealed that the two overexpressed proteins interact with each other after cell stimulation but not in resting cells (Fig. 4C). Therefore, it was important to show that the interaction occurs between the endogenous proteins as well. To this end, coimmunoprecipitation revealed that as for the overexpressed proteins, there was very little interaction in resting cells, but TPA treatment stimulated this interaction mainly 5 minutes upon stimulation (Fig. 4D). Therefore, it is likely that shortly after stimulation, ERK interacts with and is phosphorylated by CK2. The phosphorylation then allows ERK binding to Imp7, and this promotes the nuclear ERK translocation.

Ser246 phosphorylation by CK2 is sufficient for full ERK translocation, while Ser244 phosphorylation accelerates it

Our previous study using APE and EPA mutants of the ERK2-NTS suggested that phosphorylation of Ser246 is more important for ERK nuclear translocation than that of Ser244 [15]. In order to further study, the role of the distinct residues, we mutated NTS within GFP-ERK2 to APS, SPA and APA. These constructs were cotransfected into CHO cells together with the RFP-conjugated MEK1 for cytoplasmic anchoring [41, 42]. After serum-starvation, the cells were treated with TPA and the nuclear translocation of the various constructs was followed. As expected, all overexpressed ERK2 constructs were localized in the cytoplasm, but after stimulation the different construct translocated into the nucleus in different kinetics (Fig. 5A). Thus, ERK-SPS (WT) translocation was fastest, and this construct was found in the nucleus of 65% of the cells already 6 min after stimulation. ERK-APS translocated slightly slower and reached this level 12 min after stimulation. The translocation of the ERK-SPA was much slower, as 15 min after stimulation only 35% of the cells had nuclear fluorescence, and ERK-APA did not demonstrated almost any nuclear staining after stimulation. These results indicate that Ser246 is sufficient to induce full, but slower ERK translocation, while the phosphorylation of Ser244 can't do it, but rather facilitates the rate of translocations when occurs together with Ser246 phosphorylation. The differences in nuclear translocation were not due to changes in TEY phosphorylation, as under the coexpression conditions used here, the NTS mutations had only minor effects on the activating phosphorylation (Fig. 5A, lower blot). The reason that the effect of SPS mutation on TEY phosphorylation was smaller than that reported in our previous publication (6), is probably due to the overexpression of MEK1 that is probably able to overcome to some extent the compromised conformational change. Similar results to those determined by cell count were found using ImageStream as well (Fig. 5B). We then tested whether the translocation by two sites might be regulated by CK2. For this purpose, we repeated the above experiment, but stimulated the cells with TPA for 15 min in the presence or absence of TBB. As expected, most ERK-SPS (WT) and ERK-APS, small portion of ERK-SPA and essentially no ERK-APA were found in the nucleus at this time (Fig. 6). TBB pretreatment abolished most of the nuclear translocation of ERK-SPS and ERK-APS (>80%), but inhibited only ~30% ERK-SPA, and had no effect on ERK-APA localization. These results indicate that CK2 is the main kinase responsible for ERK translocation, but other kinases might participate in Ser244 phosphorylation and influence the kinetics of ERK translocation.

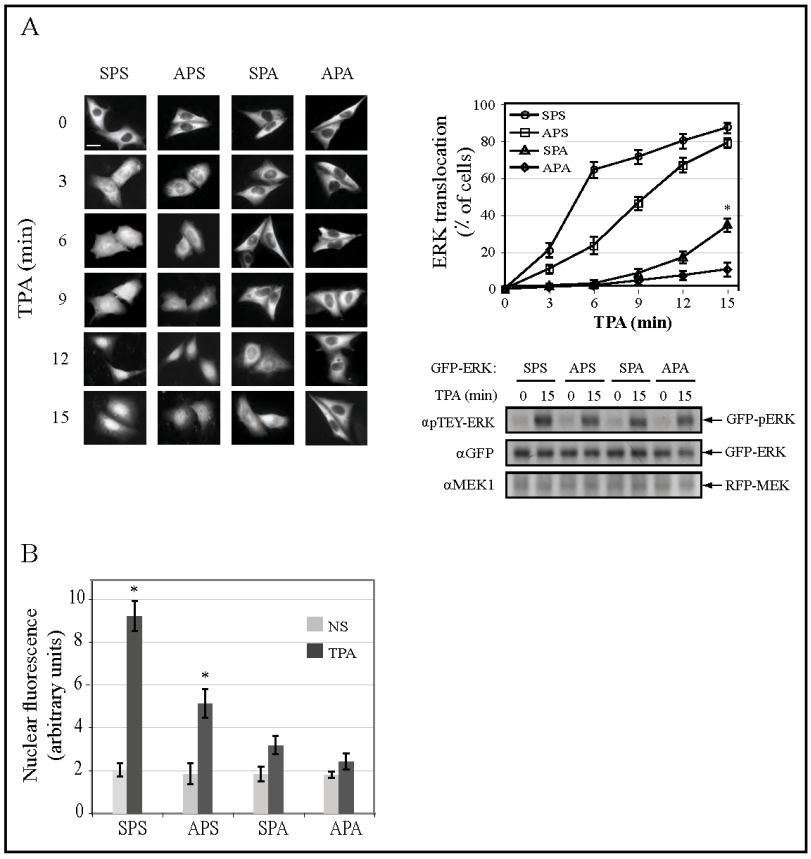

Fig. 5. Kinetics of NTS-mutated ERK translocation into the nucleus. (A) CHO cells were co-transfected with RFP-MEK1 and the indicated GFP-ERK2 constructs. Thirty-six h after transfection the cells were serum-starved and then stimulated with TPA (250 nM) for the indicated times. The cells were processed and stained as described above. Scale bar -15 μm. About 100 cells from each group in 10-12 random microscopic fields were monitored. The graph represents percent of cells in which GFP-ERK2 was mostly localized in the nucleus. The statistical difference of cells with SPA mutant compared to WT-ERK2 at 15 min after stimulation was assessed by student T-test. * P<0.01. P-ERK, as well as ERK and MEK expression was examined using whole cell extract (lower Western blots). (B) Analysis of the nuclear translocation of GFP-ERK2 mutants by ImageStream. HeLa cells were co-transfected with pcDNA-MEK1 and the indicated GFP-ERK2 constructs. Thirty-six h after co-transfection the cells were serum-starved and then stimulated with TPA (250 nM) for 6 minutes. The treated as well as the non-treated control cells (100-170 per sample) were processed as described under Material and Methods and analyzed by ImageStream. The graph represents geographic mean of nuclear ERK fluorescence ± SE. * p<0.05.

The phosphorylation of Ser244 is mediated by both CK2 and ERK

Since Ser244 is not a classical phosphorylation site of CK2, we undertook to study the mechanism of phosphorylation of this site. As CK2 was shown above to be the main responsible kinase for the translocation, we first examined the ability of CK2 itself to phosphorylate each of the Ser residues within the NTS by *in vitro* phosphorylation of WT-ERK2 and the various NTS mutants. As expected, CK2 efficiently phosphorylated the recombinant GST-ERK2-SPS, and failed to phosphorylate the ERK2-APA construct (Fig. 7A). However, the ability of CK2 to phosphorylate the various NTS mutants was compromised. Thus, the rate of phosphorylation of the predicated CK2 site (Ser246 in ERK2-APS and ERK2-EPS) was roughly half of ERK2-SPS phosphorylation by CK2. The reason for this reduced phosphorylation of Ser246 in APS or EPS is not clear but might be due to changes in ERK1/2 conformation. On the other hand, Ser244 was not phosphorylated at all when Ser246 was mutated to Ala (ERK2-SPA), but the phosphorylation was rescued when Ser 246 was mutated to Glu (ERK2-SPE). These results indicate that both Ser residues within the NTS can be phosphorylated by CK2, but Ser244 can be phosphorylated only after Ser246 phosphorylation, supporting the ability of CK2 to phosphorylate Ser residues when position +2 is primed by phosphorylation [40].

The ability of CK2 to phosphorylate both Ser244 as well as the predicted Ser246 might indicate that this kinase is sufficient to induce full phosphorylation of the NTS. However, since the results above (Fig. 6) showed that the translocation of ERK2-SPA is only partially inhibited

by TBB, we undertook to examine the possibility that this site is phosphorylated by other kinases as well. Since this site lies within an ERK consensus phosphorylation site and ERK is activated upon stimulation, we entertained the possibility that Ser244 is partially phosphorylated by this kinase as well. For this purpose, we first examined the ability of active ERK to phosphorylate the NTS *in vitro*, using immunoprecipitated WT GFP-ERK2, inactive ERK2 (TEY-AAA), and ERK2 in which the two Ser residues within the NTS were replaced with Ala (SPS-APA). These three constructs were subjected to *in vitro* phosphorylation by either GST-ERK2, which has very low activity, active GST-ERK2 (Act-ERK), or inactive GST-ERK2 (ATP binding site mutation; KA-ERK). ^{32}P incorporation into GFP-ERK2 was detected mainly when this protein was incubated with Act-ERK2. The phosphorylation of TEY-AAA was reduced by ~25%, while that of SPS-APA was reduced by ~80% (Fig. 7B). Next, we analyzed the ability of active ERK2 to phosphorylate GST-ERK2 and its NTS mutants. As expected, active ERK2 phosphorylated both ERK2-SPS and ERK2-SPA (Fig. 7C), but not ERK2-APS or ERK2-APA. These findings suggest that while Ser246 is phosphorylated mainly by CK2, Ser244 phosphorylation may be mediated by active ERK1/2 as well.

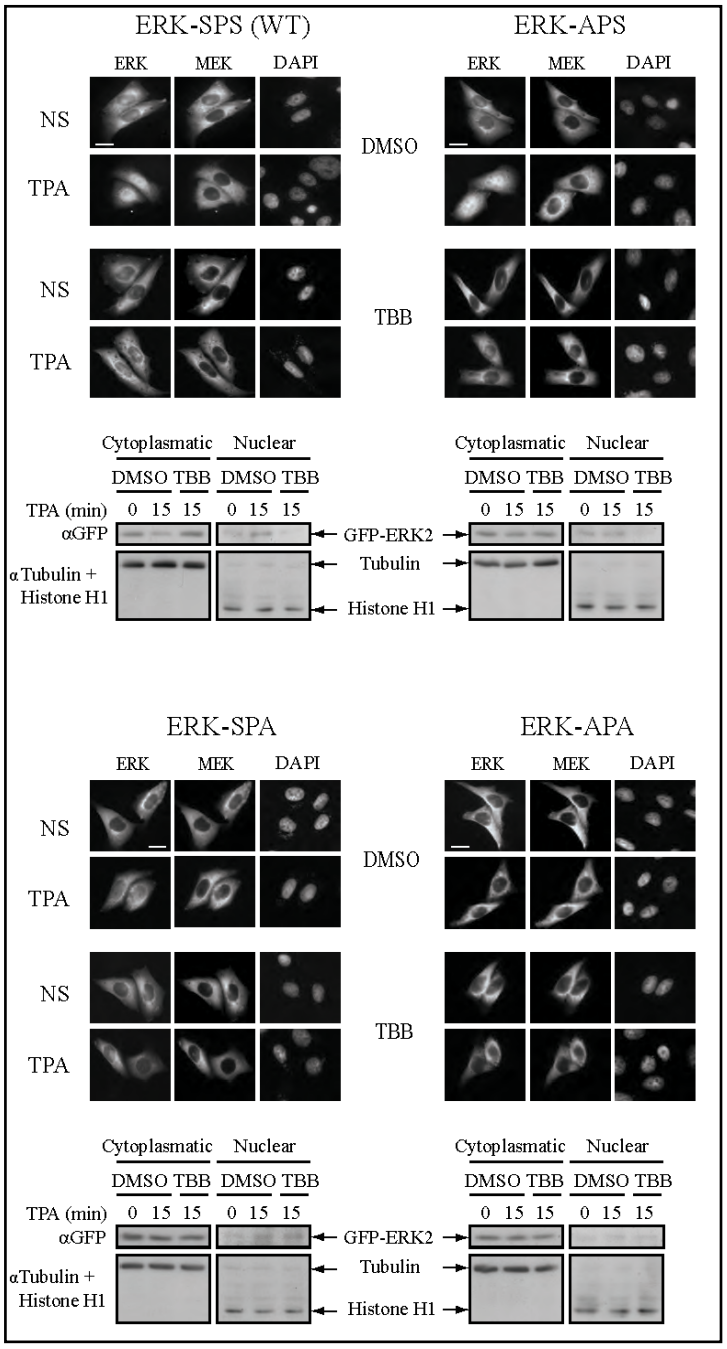

Fig. 6. Role of distinct Ser phosphorylation within the NTS in the nuclear translocation of ERK. CHO cells were co-transfected with the following GFP-ERK2 constructs: SPS (WT), APS, SPA, APA and RFP-MEK1. Thirty-six h after transfection the cells were serum starved, pretreated with either 10μM of TBB (10 μM) or DMSO control for 2 h, and then either stimulated with TPA (250 nM; 15 min) or left untreated, NS). The cells were processed and stained as in Fig.1A. Scale bar - 15nm. In the parallel experiment the cells were seperated to cytoplasmatic/nuclear fractions, and the amount of GFP-ERK2 constructs was analyzed with αGFP Ab (upper panels). Tubulin and Histone H1 served as loading control for cytosolic/nuclear fraction respectively (bottom panels).

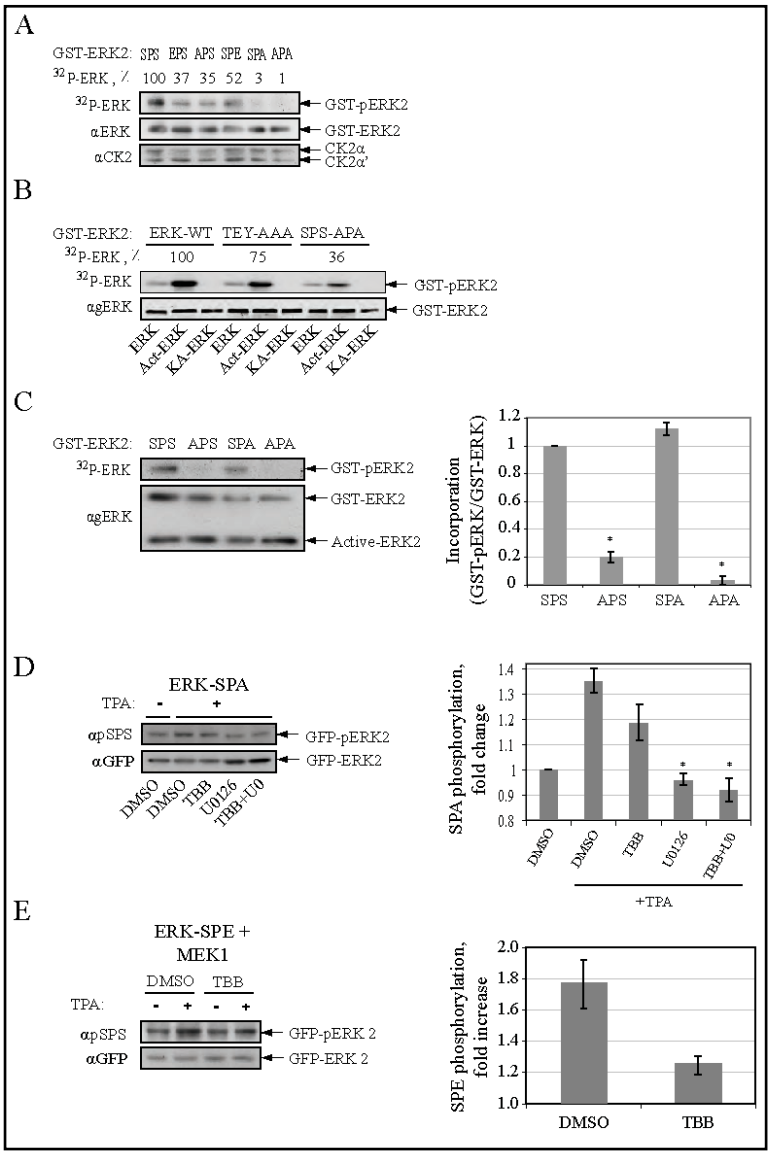

Fig. 7. CK2 phosphorylates both Ser244 and Ser246, while ERK participates in Ser244 phosphorylation. (A) CK2 phosphorylates both Ser244 and Ser246 in-vitro. The indicated GST-ERK2 constructs (0.1μg per sample) were subjected to an in-vitro CK2 phosphorylation as described. The phosphorylated proteins were detected by autoradiography, and band intensities were quantified by densitometry. The substrates and kinase were detected using the appropriate Abs. (B) ERK phosphorylates ERK's NTS in-vitro. The GST constructs ERK2-WT, ERK2-TEY-AAA or ERK-APA (SPS-APA) were subjected to in vitro phosphorylation with either GST-ERK2 (ERK), active GST-ERK2 (Act-ERK) or kinase dead GST-ERK2 (KA-ERK). The phosphorylation was detected by autoradiography. Amount of ERK was detected using αgERK Ab. The intensity of the phosphorylation (percent of ERK-WT is shown on top of upper blot) was quantified by densitometry. (C) ERK phosphorylates Ser244 in vitro. The indicated GST-ERK2 constructs (0.1μg per sample) were subjected to in-vitro phosphorylation with ERK2. The amounts of phosphorylated constructs were detected by autoradiography, and that of the GST and endogenous kinase were detected by Western blot analysis using αgERK Ab. The bar-graph represents the incorporation of ^{32}p into GST-ERK as quantified by densitometry, (* p<0.05). (D) ERK phosphorylates Ser244 in-vivo. HEK-293T cells were transfected with GFP-ERK2-SPA, serum starved and pretreated with DMSO (control), TBB (10μM), U0126 (5μM) or both inhibitors for 2 h. Then the cells were stimulated with TPA (250nM, 10 min) or left untreated. pSer244 was detected using polyclonal αpSPS Ab, and the amount of ERK was detected by αGFP Ab. The bar-graph represents SPA phosphorylation quantified by densitometry, (* p< 0.05). (E) Phosphorylation of Ser244 in-vivo is mediated mostly by CK2. HEK-293T cells were co-transfected with GFP-ERK2-SPE and RFP-MEK1, pretreated with TBB (10M) or DMSO control for 2 h, and stimulated with TPA (250 nM, 15 min). pSer244 was detected as above, and quantified by densitometry (bar-graph, * p< 0.05).

The ability of ERK to phosphorylate Ser244 *in vitro* raised the question as whether these kinases can also phosphorylate this site *in vivo*. To answer this question, we transfected the GFP-ERK2-SPA construct into HEK-293T cells, which were then serum-starved and pretreated with TBB, U0126 or both of them. Then, the cells were stimulated with TPA, and Ser244 phosphorylation of the construct was detected using special anti pSPS Ab that

recognizes mono as well as dually phosphorylated NTS. This experiment revealed that the phosphorylation of ERK2-SPA was significantly increased upon TPA stimulation (Fig. 7D). TBB had no significant effect on the Ser244 phosphorylation, while U0126 or the combination of TBB and U0126 abolished it. Since the ERK2-SPA construct can't be phosphorylated by CK2, these results indicate that Ser244 is indeed phosphorylated by ERK upon stimulation in the examined cells. To explore the relative role of CK2 and ERK in the phosphorylation of Ser244, we then transfected the cells with ERK-SPE mutant that can be phosphorylated by both kinases, pretreated them with TBB and stimulated the cells with TPA. As expected, NTS phosphorylation of this construct was elevated after stimulation, and this elevation was inhibited by TBB (~70%, Fig. 7E). Since TBB inhibited more than 95% of the CK2 activity (data not shown), the small elevation of phosphorylation is likely mediated by active ERK. Indeed, U0126 added to the TBB abolished the residual phosphorylation, indicating that Ser244 phosphorylation is mediated mainly by CK2 but also by active ERK.

The induction of CK2 phosphorylation is mediated by the release of hindered NTS from cytoplasmic anchors

Since CK2 is a constitutively active kinase whose activity is not changed upon stimulation, the reason for the significant increase in the CK2-mediated phosphorylation upon stimulation was not clear. One possibility might be that the CK2 phosphorylation site is hindered in resting cells and is exposed only after stimulation. Indeed, in resting cells, ERK interacts with various anchoring proteins, and the interaction with many of them is reversed upon stimulation and TEY phosphorylation [43]. Therefore, we undertook to study whether MEK1, which serves as one of the cytoplasmic anchors, prevents the phosphorylation of NTS by CK2. First we confirmed that MEK1/2 serve as anchor proteins, and found that knocking down these kinases indeed causes nuclear translocation of about 20-25% of the ERK molecules (Fig. 8A). In addition, MEK1/2 knockdown elevated NTS phosphorylation in quiescent but not stimulated cells. These results indicate that endogenous MEK1/2 are responsible for the anchoring of a portion of ERK1/2 molecules, which upon release from the MEK1/2 are phosphorylated on their NTS by CK2. To further establish it, we examined the ability of CK2 to phosphorylate GST-ERK2 in the presence of increasing concentrations of His-MEK1. We found that the NTS phosphorylation by CK2 was decreased upon increasing HA-MEK1 concentration (Fig. 8B). This effect was not due to inhibition of CK2 activity, because the latter had no effect on the phosphorylation of casein in a similar experiment. Thus, it is likely that MEK1 hinders the NTS, and protects it from CK2 phosphorylation. Finally, we used the CRS-AAA that does not interact with most of the anchoring proteins [42]. When this construct was transfected together with MEK1, its basal CK2-dependent NTS phosphorylation was much higher than WT (Fig. 8C), and this phosphorylation was prevented by TBB. These data clearly suggest that the NTS is hindered by anchoring proteins and is released upon stimulation to allow its phosphorylation by CK2.

The SPE mutant exhibits an increased electronegative patch on the protein surface

Structural comparison of ERK2-SPE and the ERK2-SPS (WT) revealed no significant difference in their interlobe orientation. When observing the Ser-246 area in the WT structure two negatively charged amino acids, Glu-248 and Asp-249 were located on the protein surface. In the SPE mutant structure Glu-246 is in close proximity of these amino acids making this region more electronegative (Fig. 9A, Table 1). Recently, the mechanism enabling substrate recognition in CK2 was discovered [44]. Crystal structure of human CK2 (PDB code: 2PVR) was solved exhibiting sulfate ions in the substrate-binding pocket mimicking the P+1 and P+3 acidic residues. This structure revealed that the first P+1 sulfate is stabilized by interaction with amino acids Arg-191 and Lys-198, while the P+3 sulfate is stabilized by interactions with Arg-155, Asn-189 and Arg-80. The conserved catalytic Asp-156 (CK2 numbering) positioned 11.3Å from the P+3 sulfate [44]. When observing the NTS studied here, the P+1 site is occupied by a polar residue, Gln-247 followed by two acidic amino acids Glu-248 and Asp-249, which we showed to be important for the phosphorylation

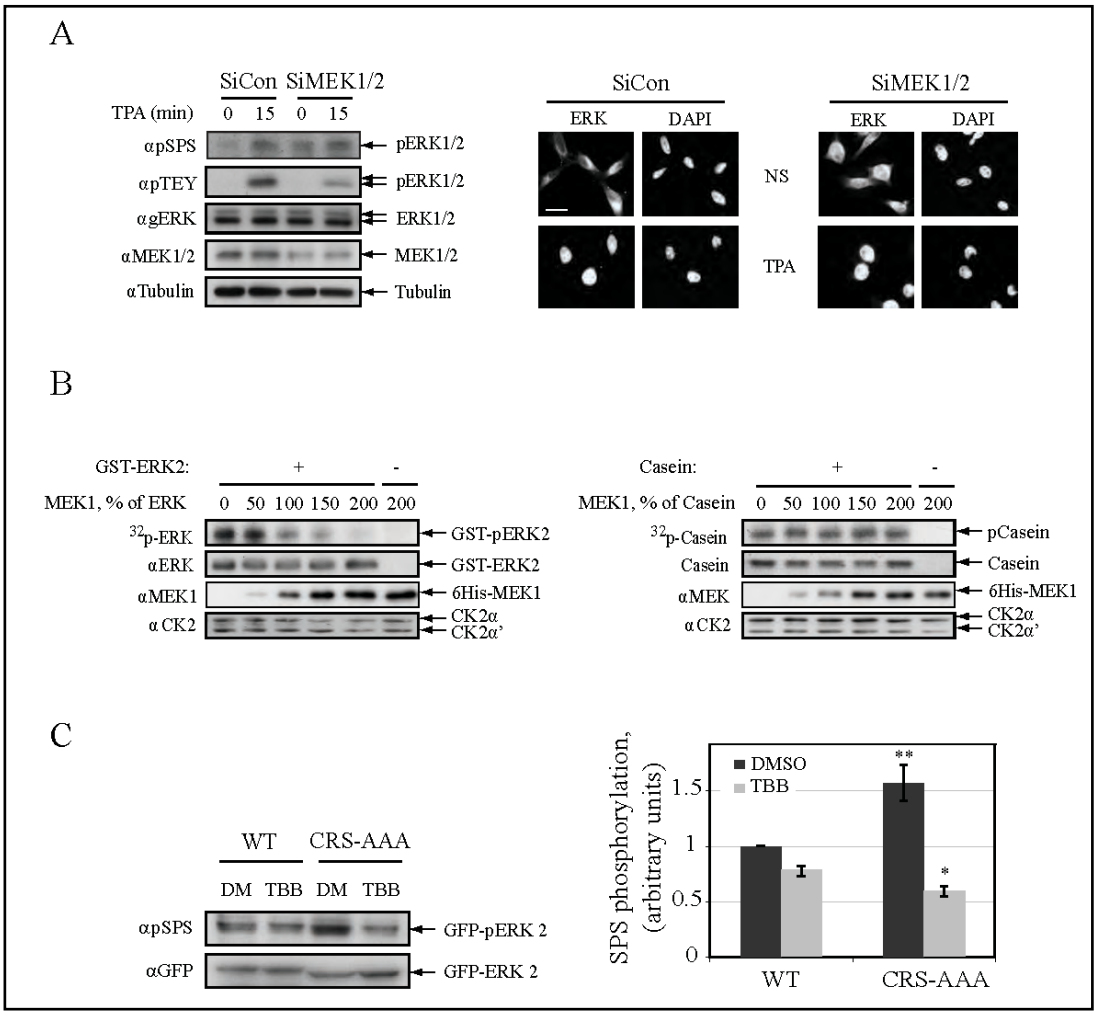

Fig. 8. The induction of CK2 phosphorylation is mediated by the release of NTS from cytoplasmic anchors. (A) Knockdown of MEK1/2 induces partial nuclear translocation and NTS phosphorylation of ERK1/2. HeLa cells were transfected with either Si-RNA of MEK1/2 or Si-RNA control and then were either stimulated with TPA (250 nM, 15 min) or left untreated as control. The cells were next harvested and their extracts were subjected to Western blot analysis using the indicated Abs. In parallel HeLa cells were grown on coverslips followed by their transfection with Si-RNA as above. The cells were then treated as above and the nuclear translocation of their endogenous ERK1/2 was analyzed in non-stimulated cells as in Fig. 1A. Scale bar – 10 μm. (B) MEK1 prevents ERK2 phosphorylation by CK2. GST-ERK2-WT (0.1 μg per sample) was mixed in the indicated proportions with 6His-MEK1 and subjected to in-vitro CK2 phosphorylation. As a control, dephosphorylated α-Casein was mixed with 6His-MEK1 instead of ERK2. GST-ERK2 and α-Casein phosphorylation was detected by autoradiography, and the amount of the indicated proteins was detected using the appropriate Abs or by coomassie staining. (C) ERK anchoring prevents phosphorylation of its NTS by CK2 in vivo. HEK 293T cells were co-transfected with either GFP-ERK2-WT or GFP-ERK-CRS-AAA together with RFP-MEK1. After serum-starvation the cells were pretreated with TBB (10μM) or DMSO control for 2 h. Cell extracts were subjected to Western blot analysis with αpSPS-ERK and αGFP Abs. The bar-graph represents NTS phosphorylation measured by densitometry (3 experiments). The statistical differences were analyzed by student T test. (bar-graph; * P<0.01).

by CK2. The distance between the two sulfates ions in the CK2 structure was 10.7Å while the in the WT-ERK2 structure, the distance between Gln-247 and Asp-249 (P+1 and P+3 site) was 11.3Å and the distance between Glu-248 and Asp-249 (the two acidic residues) was 4.72Å, both distances are not suitable for the substrate binding pocket. However, the

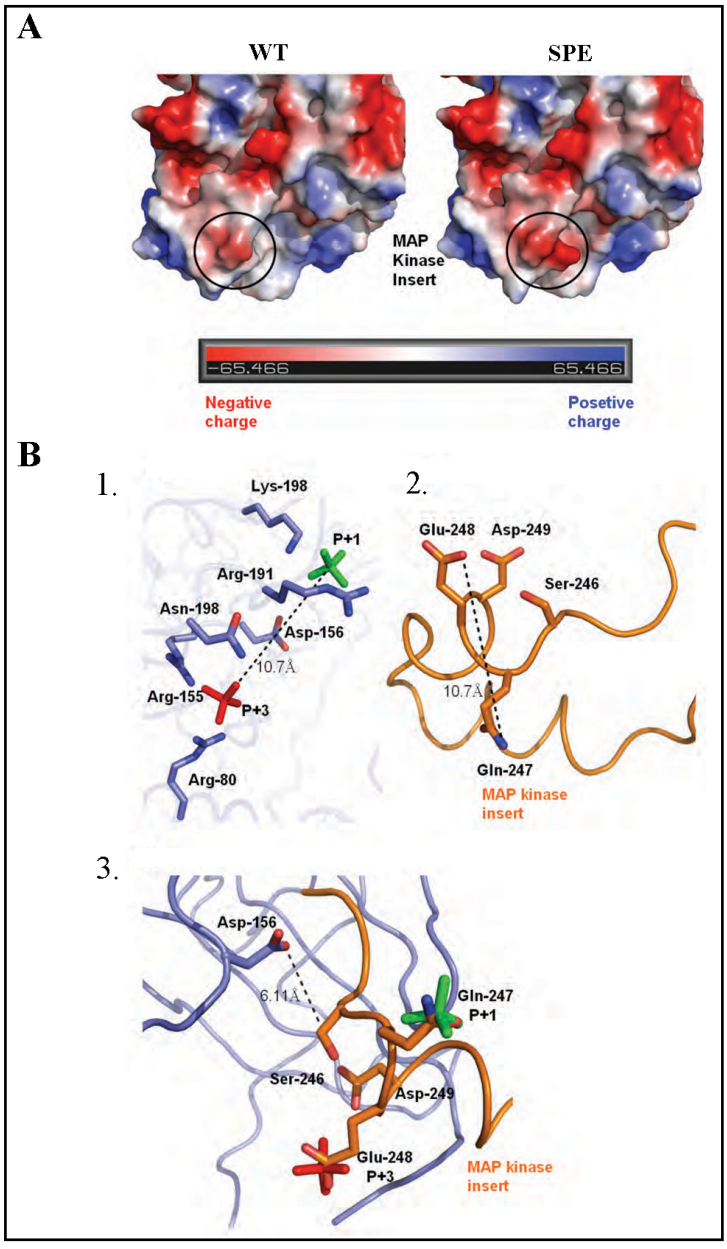

Fig. 9. Structural analysis of SPE-ERK2. (A) Electrostatic surface presentation of WT-ERK2 and SPE-ERK2 of the C'-lobe. The SPS region (circled) display increased electonegativity due to the S246E mutation, which could have an effect on importin7 recognition. (B) ERK2- CK2 binding model. (I) The available crystal structure of human CK2α (PDB code: 2PVR) (blue) with two sulfate ions in the P+1 site (green) and P+3 site (red) are located in a 10.7Å apart. Amino acid important for substrate recognition pocket are shown and labeled. (II) The MAP kinase insert of the SPS region of ERK2 and the side chains of Ser-246, Gln-247, Glu-248, and Asp-249 are shown in orange (orange). The distance between the side chains of Gln-247 and Glu-248 is measured to be 10.7Å similar to that of sulfate ions in the CK2 active site. (III) The structures of ERK2 and CK2 were pair fitted on Gln-247 and Glu-248 (ERK2) to the P+1 and P+3 sulfate ions from CK2 which exhibit an almost perfect fit introducing Ser-246 into the active site (note that no calculation was conducted). In this regard, Ser-246 Oγ is located 6.11Å from Asp-156 Oδ1 yet with minimal conformational change is the side chains conformation a distance acceptable for polar interaction could be reached.

distance between Gln-247 and Glu-248 was exactly right 10.7Å. We have generated an initial molecular model while pair fitting Gln-247 and Glu-248 from WT-ERK2 to the P+1 and P+3 sulfate ions from CK2 structure respectively (Fig. 9B and Table 1). Gln-247 and Glu-248 were stabilized by the same interaction described for the sulfate ions in the CK2 structure. Ser-246 is in close proximity to the catalytic Asp-156 (a distance of 6.11Å), indicating Gln-247 and Glu-248 to be the important residues for proper interaction between ERK2 and CK2.

Discussion

Recently, we have elucidated a novel mechanism of stimulated nuclear translocation, which involves NTS phosphorylation and binding to importin7 [15]. As of today, this NTS has been identified in ERK, MEK1, SMAD3, the Drosophila protein Drosha [45], but is likely to mediate the nuclear translocation of other signaling proteins as well [46]. Moreover, role of

importin7 in the translocation of signaling molecules was shown in stimulated SMAD [47] and JUN [48]. In the current study, we extended our knowledge of the NTS by showing that its phosphorylation is mediated by CK2 and to some extend by trans-autophosphorylation. We also found that in resting cells, this phosphorylation is prevented due to sequestering interactions with cytoplasmic anchoring proteins, which are released upon stimulation. Our results best fit a model in which ERK is localized in the cytoplasm of resting cells due to interaction with various proteins [49]. These proteins anchor ERK by interaction with a region that contains the NTS of ERK, which is therefore hindered. Upon stimulation, the regulatory Thr and Tyr within the activation loop of ERK are phosphorylated, which induces their release from the anchoring proteins [43]. Consequently, the NTS of ERK is exposed (Fig. 8) and can undergo phosphorylation on its two Ser residues (Fig. 2). Ser 246 seems to be phosphorylated mainly by CK2, while Ser244 phosphorylation is mediated by both CK2 (~ 70%) and activated ERK (~30%; Fig. 7). Phosphorylation of Ser246 is sufficient to induce full (but slower) nuclear translocation of ERK, while the phosphorylation of Ser244 accelerates it, but can't induce significant translocation by itself (Fig. 5). The phosphorylation then forms an acidic patch (Fig. 9) that induces binding of ERK to importin7 and consequently allows nuclear shuttling through the nuclear pores.

The determination of signaling specificity of the ERK cascade has attracted much attention over the past years [50]. One of the main parameters that controls ERK's selectivity is the subcellular distribution of this kinase as well as its upstream regulators [2]. In this sense it has previously been shown that the nuclear activity of ERK is important for the activation of transcription factors necessary for relevant gene expression [9]. Other roles of the nuclear activities, such as chromatin remodeling, direct induction or suppression of transcription and regulation of cell cycle, have been demonstrated as well. Therefore, nuclear translocation of ERK is essential for the induction of many ERK dependent-processes, and indeed, specific abrogation of ERK nuclear translocation blocks the progression of proliferation and oncogenic transformation [51-53]. It should be noted however, that the nuclear activity is probably not sufficient to induce all ERK-dependent processes, as their activity in the cytoplasm [54] and mitochondria [55] as well as ERK1c's activity in the Golgi [56, 57] are also necessary to induce proliferation and survival. Therefore, the regulators of ERK translocation seems to be regulated by variety of methods such as: cytoplasmic anchoring by interaction with specific proteins [49, 58], changes in NPCs' number in different cell type [59], interaction with importin7 [60] and phosphorylation by CK2 (here). All these mechanisms are likely to play important role in governing the specificity and efficiency of ERK signaling in health and disease.

ERK plays a key role in regulating cell cycle progression upon extracellular stimulation, which enhances proliferation and may lead to oncogenic transformation [61]. Since CK2 is the main kinase that phosphorylates the NTS of ERK, it was likely to assume that it participates in the regulation of these processes as well. Indeed, it was clearly shown that CK2 participates in the regulation and progression of several stages of the cell cycle [24]. This was verified in several systems including mainly mammalian cells, in which reduction of CK2 expression or activity inhibits G0/G1, G1/S, and G2/M transitions [19]. Indeed, addition of CK2 inhibitors or knocking down CK2 in our cellular system attenuated cell proliferation. Although much information on the involvement of CK2 in proliferation is already available, the regulatory mechanisms coordinating its numerous functions are not clear enough. In this sense it is possible that our identification of ERK-CK2 cooperation might be one of the ways by which CK2 exert its function on cell proliferation. In addition to the role of CK2 in proliferation, it was also shown to participate in the induction of oncogenic transformation and tumor maintenance [25]. However, studies in experimental transgenic mice models suggested that CK2 itself is not an oncogene but cooperates with oncogenes as well as pro-tumorogenic and signaling molecules, thus increasing their oncogenic potential [62, 63]. Some studies indicate that CK2 may cooperate with the ERK cascade in the promotion of tumorogenity. For example, it was shown that CK2α' catalytic subunit synergized with H-Ras in BALB/c 3T3 fibroblasts transformation [64]. In addition, CK2 promoted transformed phenotype

and survival through Her-2/neu signaling in NF639 breast cancer cells [65]. Although the mechanisms suggested in the above studies did not include ERK, the well-known involvement of the latter in oncogenic transformation may suggest that at least part of the effect might involve CK2-regulated nuclear accumulation of ERK described here.

The involvement of CK2 in the phosphorylation of both Ser residues in the NTS has another implication in our understanding of both passive and active translocation of ERK [66]. Since CK2 is a constitutively active kinase, it may phosphorylate the NTS whenever this region is not hindered. Although in resting cells most of the ERK molecules are attracted to anchoring proteins that hinder the SPS, some of the attached through distinct sites [67] that probably do not cover the NTS. These non-hindered NTS regions may be phosphorylated by CK2 at any time and therefore may explain the relatively high basal NTS phosphorylation in resting cells, which results in a relatively small induction in this phosphorylation. In addition, it may explain the free nuclear shuttle of overexpressed proteins that are mostly free of anchoring interaction [42], and therefore, their NTS is accessible for the constitutively phosphorylation by CK2. Another interesting issue that resulted from our study is related to the consensus phosphorylation site of CK2. This site was traditionally thought to include an acidic amino acid, 3 residues C-terminal to the phosphorylated Ser/Thr (position +3), while other acidic residues in the vicinity of the Ser/Thr were thought to accelerate the rate of phosphorylation [39, 40]. Here we show that aside of the canonical Glu at position +3, phosphorylated amino acid or acidic residue at position +2 may dictate the phosphorylation by CK2 (Ser244). Therefore, these results may expend the knowledge on CK2 phosphorylation in various unknown substrates.

In summary, we found that ERK's NTS is phosphorylated by CK2, demonstrating for the first time the cross-talk between them. Unexpectedly, CK2 phosphorylates not only Ser246 within its consensus site, but after initial Ser246 phosphorylation, also Ser244. Ser244 is phosphorylated by activated ERK as well. We further found that Ser246 phosphorylation is sufficient to induce slow nuclear translocation, while pSer244 can't induce this translocation by itself, but may accelerate the pSer246 effect. Binding of inactive ERK to anchoring proteins (e.g. MEK1) hinder the NTS, and upon stimulation, the NTS is released to allow their phosphorylation by CK2 and ERK. Finally, we crystallized the phosphomimetic mutants of ERK2, and found that they form a strong electronegative patch in the KID of ERK2, which was shown by mutations to participate in the interaction of ERK with importin7. Overall, our results provide a new insight into three distinct signaling problems. First, we shed light on the mechanism of ERK translocation into the nucleus. Second, we provide new understanding of CK2 activity by: (i) demonstrating cooperation of CK2 with ERK in regulating nuclear activities, and (ii) identifying an unexpected phosphorylation site of CK2 (Ser244). Finally, we provide new data regarding the binding of importin7 to its cargo proteins.

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Disclosure Statement

The authors report no conflict of interest.

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