Lfc phosphorylation sites: identification and physiological relevance

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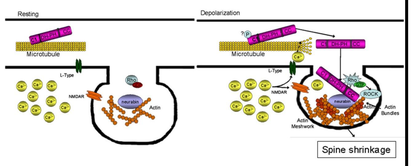
INTRODUCTION

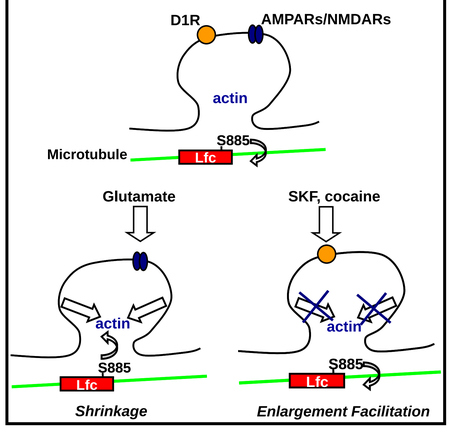
Spine morphology is thought to play an important role in determining the quality of the integration of neurotransmission in the brain, and the regulation of the F-actin cytoskeleton in spines is thought to be critical for the control of spine shape and size. Lfc is a Rho guanine exchange factor (GEF) enriched in the nervous system which has been shown to translocate from the dendritic shaft of the neuron to spines following neuronal stimulation (Ryan et al., 2005). Within spines, local activation of Rho by Lfc has been found to reduce spine size presumably via through control of actin dynamics. We hypothesized that the mechanism of shaft-to-spine translocation of Lfc may involve phosphorylation of Lfc. Therefore, we tried to identify the different phosphorylation sites of this protein, as well as their physiological relevance. In this poster, we will only address the identification and regulation of the Ser885 phosphorylation site.



Ryan et al, Neuron, 2007

MODEL

Phosphorylation of Ser885 has been shown to facilitate association of GEF-H1 (human form of Lfc) with microtubules, at least indirectly and decrease its Rho-GEF activity (Zenke et al., 2004). The presence of Lfc in the spine induces spine shrinkage (data not shown). Therefore, we propose that phosphorylation of the Ser885 residue protects the spine from shrinkage. Furthermore, since we have shown that a D1 stimulation enhances phosphorylation of Ser885, Lfc could be trapped outside the spine, with a reduced RhoGEF activity, preventing it to induce spine shrinkage. This could be a LTP facilitation step for the spine responding to a D1 stimulation.



CONCLUSION

We have used mass spectrometry to identify five phosphorylation sites in Lfc: Ser143, Ser177, Ser646, Ser885 and Ser931. One of these sites, Ser885, was found to be phosphorylated by protein kinase A. In striatal slices SKF81297, a dopamine, D1 receptor (D1R) agonist, increases Ser885 phosphorylation. Moreover, cocaine treatment of mice *in vivo* increases phosphorylation of Lfc at this site in striatal samples. Given the action of D1R on spine morphogenesis in response to cocaine exposure, phosphorylation of Ser885 of Lfc may be involved in the actions of dopamine on actin dynamics and control of spine shape, in D1R-containing neurons.

RESULTS

1. Identification of Lfc phosphorylation sites

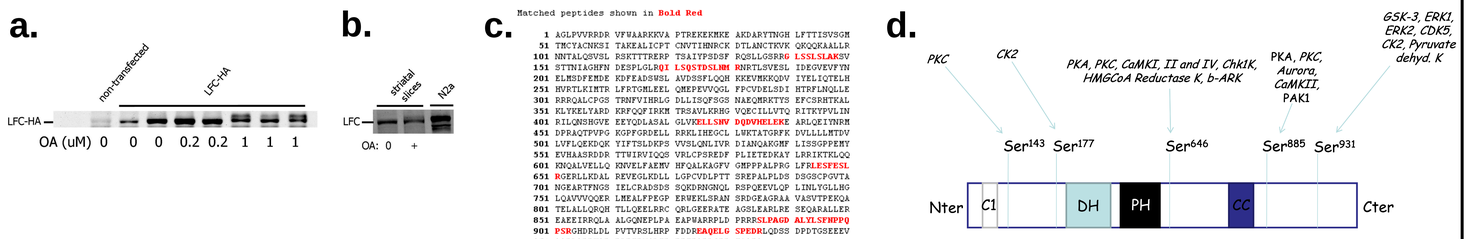


Figure 1a.: HEK cells were transfected with Lfc-HA and treated 24 hrs later with Okadaic Acid for 1 hour (OKA 0.2 μM to inhibit PP2A, or 1 μM to inhibit both PP1 and PP2A). Lfc-HA was then immunoprecipitated and analyzed by SDS-PAGE. We observed a shift when using a 1 uM dose of OKA. **Figure 1b**.: The same shift was observed in studies using striatal slices. The shift observed in both 1a. and 1b, is probably due to a phosphorylation. Figure 1c. and Figure 1d.:The same experiment as in 1a. was carried out using 20 fold cell extract in order to perform mass spectrometry on Lfc. Five phosphorylation sites were identified: Ser143, Ser177, Ser646, Ser885 and Ser931. Their localization in Lfc peptidic sequence (**Figure 1c**.) and their putative kinases (in italic, **Figure 1d**.) are shown.

2. Example of Ser885: the Western-Blot approach

Figure 2a: Lfc was expressed in HEK cells and cells then treated 24 hrs later with okadaic acid (OKA, 1 μM for 1 hour). After lysing the cells, Lambda phosphatase (PPase) was added in the total lysate for 30 min. The cell lysate was then analyzed by SDS-PAGE and immunoblotting with RU1634 (antibody against phosphoSer-885). No signal for phosphoSer-885 was found after PPase treatment suggesting that the antibody was phospho-specific. **Figure 2b**.: Both Lfc-WT and Lfc-Ser885Ala were expressed in HEK cells, and cells treated without or with the PKA activator forskolin (Fsk, 10 μM, 10 min). Immunoblotting revealed that Ser-885 phosphorylation was stimulated by forskolin and confirmed the phospho-specificity of the antibody.

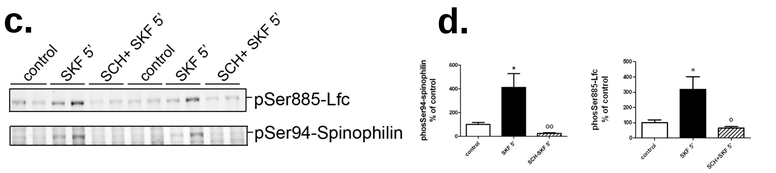


Figure 2c: Striatal slices were treated without or with the dopamine D1 receptor agonist SKF81297 (10 μM) or with the D1 receptor antagonist SCH23390 (added 30 min before). Slices were then sonicated and analyzed by SDS-PAGE and immunoblotted for Ser-885 of Lfc, or phospho-Ser94 of spinophilin (another site phosphorylated by PKA). **Figure 2d**.: quantification of two experiments.

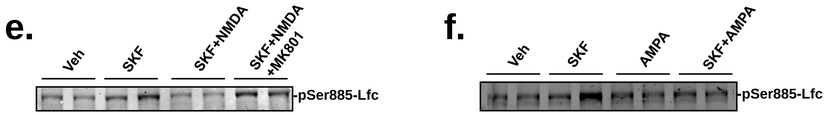


Figure 2e and 2f: Striatal slices were treated without or with SKF81297 (10 μM), in combination with NMDA (100 μM), MK801 (10 μM, added 30 min before) or AMPA (50 μM). Slices were then sonicated and analyzed by SDS-PAGE and imuunoblotted for Ser-885 of Lfc

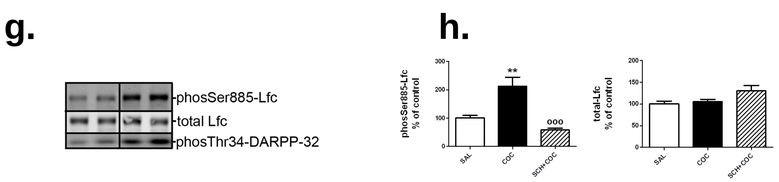
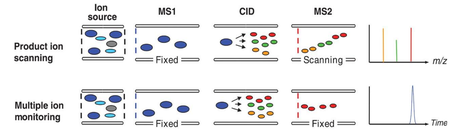
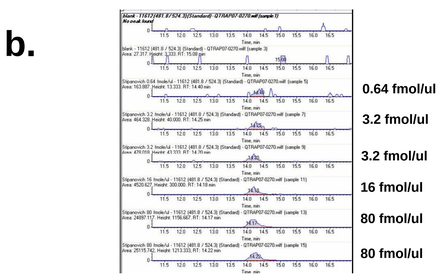


Figure 2g: Mice were injected a single dose of cocaine (20 mg/kg) and after 10 min were decapitated and the brains frozen rapidly in liquid nitrogen. Striatal samples were then sonicated and analyzed by SDS-PAGE and immunoblotted for Ser-885 of Lfc, total Lfc or anti-phosphoThr34 of DARPP32 as a positive control. **Figure 2h**: Quantification of multiple experiments (n=7).

3. The MRM approach





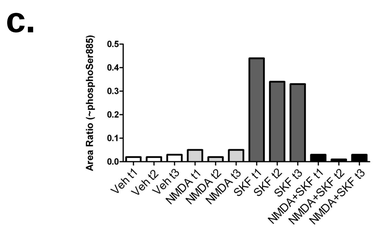


Figure 3a. Schematic representation of various types of tandem mass spectrometry experiments. Multiple reaction monitoring (MRM) consists of a series of short experiments in which one precursor ion and one specific fragment characteristic for that precursor are selected by MS1 and MS2, respectively. Typically, the instrument cycles through a series of transitions (precursor-fragment pair) and records the signal as a function of time (chromatographic elution). MRM is used for the detection of a specific analyze with known fragmentation properties in complex samples (from Domon and Aebersold, Science 2006). **Figure 3b.** Different concentrations of heavy phosphopetides were doped into the sample in order to measure the amount of each peptide. **Figure 3c.** Striatal slices were treated with the same conditions as in **Figure 2e.** and processed by MRM. Three separate MRM transitions (t1, t2 and t3) were analyzed on each sample from each condition, and the area ratio corresponding to the level of phospho-Ser885 is shown.

METHODS

Phosphorylation of Lfc in HEK 293T Cells - Full-length Lfc was transiently expressed in 293T cells for 36 h following Lipofectamine2000 transfection. Cells were treated with forskolin (50 μM) for 5 min and lysed in 1% SDS containing protease inhibitors and phosphatase inhibitors. Equal amounts of total protein were resolved on 4-12% acrylamide gels and analyzed by immunoblotting.
Generation of Phosphorylation State-specific Antibodies - Rabbit polyclonal phosphorylation state-specific antibodies were generated and purified essentially as described (Czernik, A. et al. 1997). Antibodies were raised against the following cysteine-containing phosphopeptides conjugated to thyroglobulin: VRL(pS)LPRAC (residues 91–98), CLPRAS(pS)LNE (residues 95–103), and CQERA(pS)LQDRK (residues 173–182). Affinity purification was performed using dephospho- and phosphopeptides coupled to SulfoLink gel (Pierce, Rockford, IL).
Treatment of Neostriatal Slices and Immunoblotting - Neostriatal slices were dissected and incubated in fresh Krebs-HCO buffer at 30°C under constant oxygenation. After 30 min, the slices were incubated in Krebs-HCO buffer in the absence or presence of the indicated drugs for 5-60 min. The slices were then frozen in liquid nitrogen and stored at 80°C until assayed. Frozen tissue samples were sonicated in boiling 1% SDS containing protein phosphatase and protease inhibitors. The protein concentrations of the homogenates were determined using the BCA protein assay method (Pierce, Rockford, IL) with bovine serum albumin as a standard. Equal amounts of protein (40 μg) were separated by SDS-PAGE using 4-12% acrylamide gels and transferred to nitrocellulose membranes (0.2 μm). Membranes were blocked for 1 h in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% (w/v) nonfat dry milk and 0.1% Tween 20 and then incubated with polyclonal antibodies against Lfc (guinea-pig or goat), phospho-Ser-885 Lfc, phospho-Ser-94 spinophilin, phosphoThr34 DARPP-32. Following washes with TBS containing 1% nonfat milk, 0.1% Tween 20, and 0.5% bovine serum albumin, proteins were visualized Chemiluminescence was detected using the Odyssey LI-COR scanner. Data were analyzed by the ANOVA test, with significance defined as $p < 0.05$.
In Vivo Protein Phosphorylation - After having been habituated to injection by saline i.p. administration during the 3 days preceding the experiment, mice were injected with saline or cocaine (20 mg/kg). At 15 min after treatment, the animals were decapitated and their head was immediately frozen in liquid nitrogen (12 s) (Pascoli *et al.*, 2005). The frozen heads were then sliced with a cryostat (210- m-thick) and five microdisks (1.4-mm diameter) were punched bilaterally from the dorsal striatum and stored at -80°C. Micropunchs were rapidly homogenized by sonication in a 1% SDS (w/v) solution containing 1 mM sodium orthovanadate (preheated at 100°C) and placed at 100°C for 5 min. Equal amounts of micropunch lysates (40 g) were separated by SDS–polyacrylamide gel electrophoresis (4-12%) and immunoblotting was performed as previously described.
Drugs – SKF81297, SCH23390, NMDA, AMPA and MK-801 were from Sigma; okadaic acid was from Tocris.
In gel Protein Digestion - Proteins isolated from 1 or 2 dimensional polyacrylamide gels were subjected to in situ enzymatic digestion. The gel pieces were washed with 250 µl 50% acetonitrile/50% water for 5 min followed by 250 µl of 50 mM ammonium bicarbonate/50% acetonitrile/50% water for 30 min. One final wash was done using 10 mM ammonium bicarbonate/50% acetonitrile/50% water for 30 min. After washing, the gel pieces were dried in a Speedvac and rehydrated with 0.1 µg of either modified trypsin (Promega), endopeptidase Lys-C (Wako), endoproteinase Glu-C (Roche) or chymotrypsin (Roche) per (approximately) 15 mm3 of gel in 15 µl 10mM ammonium bicarbonate. Samples are digested at 37°C for 16 hours.
Titanium dioxide enrichment - The digests were acidified with 0.5% TFA, 50% acetonitrile. Top Tips (Glygen Corp.) were prepared by washing 3 times with 40 µl of each 100% acetonitrile, followed by 0.2 M sodium phosphate pH 7.0, and 0.5% TFA, 50% acetonitrile. Washes were spun through into an eppendorf tube at 2,000 rpm for 1 min. The acidified digest supernatant was loaded into the TopTip, spun at 1,000 rpm for 1 min, and then 3,000 rpm for 2 min. Gel pieces were rinsed with 40 µl 0.5% TFA, 50% acetonitrile, and the supernatant transferred to the Top Tip and the spin repeated. The Top Tip was then washed with 40 µl 0.5% TFA, 50% acetonitrile and the spin repeated. The flow through from these washes were saved and analyzed by LC-MS/MS as below. Phosphopeptides were eluted from the TopTip 3 times with 30 µl 28% ammonium hydroxide. Both the flow through and eluted fractions were speedvaced to dryness, and re-dried from 40 µl of water. Samples were dissolved in 3 µl 70% formic acid, vortexed, diluted with 7 µl 0.1% TFA, spun and transferred to LC-MS/MS vials where 5µl was used for MS analysis.
LC-MS/MS on the LTQ Orbitrap - The LTQ Orbitrap was equipped with a Waters nanoAcquity UPLC system, and used a Waters Symmetry® C18 180 µm x 20 mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35°C) for peptide separation. Trapping was done at 15µl/min, 99% Buffer A (100% water, 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nl/min with Buffer A: 100% water , 0.1% formic acid and Buffer B: 100% CH3CN, 0.075% formic acid. A linear gradient (51 min) was run with 5% buffer B at initial conditions, 50% B at 50 minutes, and 85% B at 51 min. MS was acquired in the Orbitrap using 1 microscan, and a maximum inject time of 900 followed by four data dependent MS/MS acquisitions in the ion trap. Neutral loss scans (MS3) were also obtained for 98.0, 49.0, and 32.7 amu. The data was searched using Mascot Distiller and the Mascot search algorithm.
Database searching - All MS/MS spectra were searched in-house using the Mascot algorithm (Hirosawa et al, 1993, version 2.2.0) for un-interpreted MS/MS spectra after using the Mascot Distiller program to generate Mascot compatible files. The Mascot Distiller program combines sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 were preferentially located with a signal to noise ratio of 1.2 or greater and a peak list was generated for database searching. Using the Mascot database search algorithm, we considered a protein identified when Mascot listed it as significant and more than 2 peptides matched the same protein. The database searched wa typically the NCBInr which is chosen over genome specific databases since a match to the correct species has more significance in the larger databases and, for some incomplete genomes, a match may be found based on homology to another species. The Mascot significance score match is based on a MOWSE score and relies on multiple matches to more than one peptide from the same protein. Parameters used for searching were partial methionine oxidation and carboxamidomethylated cysteine, a peptide tolerance of ±20ppm, MS/MS fragment tolerance of ±0.6 Da, and peptide charges of +2 or +3. Normal and decoy database searches were run.
Quantitative mass spectrometry via MRM - LC-MRM was carried out on an ABI 4000 QTRAP triple quadrupole mass spectrometer interfaced with a Waters nanoAcquity UPLC system running Analyst 1.5 software. Proteins were prepared for MS as described above. Dried digests were resuspended in 3 µL 70% formic acid (FA) and brought to a final volume of 15 µL in 0.1% FA. A typical MRM scan was carried out by loading 10 µL of sample onto a 180 µm x 20 mm 5µm Symmetry C18 nanoAcquity trapping column with 2% acetonitrile (ACN)/0.1% FA at 15 µL per minute for 3 min. After trapping, a 2-40% 30 min linear ACN/0.1% FA gradient, was run at flow rate of 0.75 µL/min with a 75 µmx100 mm 1.7 µm BEH130 C18 nanoAcquity column. MRM scanning for each LC-MRM experiment was carried out with 47 transitions and a cycle time of 4.935 sec with a 100 millisec dwell time per transition. The following transitions were used for quantitation of Lfc peptides:
GL(13C6)(15N1)SS(PO4)LSLAK/GLSS(PO4)LSLAK/ L(13C6)(15N1)ES(PO4)FESLR/ LES(PO4)FESLR/ELLSNVDQDVHEL(13C6)(15N1)EK/ELLSNVDQDVHELEK/ EAQEL(13C6)(15N1)GS(PO4)PEDR/EAQELGS(PO4)PEDR/QIL(13C6)(15N1)SQSTDS(PO4)LNM R/QIL(13C6)(15N1)SQSTDS(PO4)LNMR/S(PO4)L(13C6)(15N1)PAGDALYLSFNPPQPSR
Transitions were selected from discovery data from the Thermo Orbitrap LTQ Mass spectrometer and refined with Applied Biosystems MRMPilot 0.9beta. LC-MRM data was processed and quantitated using Applied Biosystems Multiquant 1.1 software. Data results from Multiquant were exported and uploaded into the Yale Protein Expression Database (YPED)(Shifman et al 2007) .

GRANT

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