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Quantitative analysis of a phosphoproteome readily altered by the protein kinase CK2 inhibitor quinalizarin in HEK-293T cells☆

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ABSTRACT

CK2 is an extremely pleiotropic Ser/Thr protein kinase, responsible for the generation of a large proportion of the human phosphoproteome and implicated in a wide variety of biological functions. CK2 plays a global role as an anti-apoptotic agent, a property which is believed to partially account for the addiction of many cancer cells to high CK2 levels. To gain information about the CK2 targets whose phosphorylation is primarily implicated in its pro-survival signaling advantage has been taken of quinalizarin (QZ) a cell permeable fairly specific CK2 inhibitor, previously shown to be able to block endogenous CK2 triggering an apoptotic response. HEK-293T cells either treated or not for 3 h with 50 μM QZ were exploited to perform a quantitative SILAC phosphoproteomic analysis of phosphosites readily responsive to QZ treatment. Our analysis led to the identification of 4883 phosphosites, belonging to 1693 phosphoproteins. 71 phosphosites (belonging to 47 proteins) underwent a 50% or more decreased occupancy upon QZ treatment. Almost 50% of these fulfilled the typical consensus sequence recognized by CK2 (S/T-x-x-E/D/pS) and in several cases were validated as *bona fide* substrates of CK2 either based on data in the literature or by performing *in vitro* phosphorylation experiments with purified proteins. The majority of the remaining phosphosites drastically decreased upon QZ treatment display the pS/T-P motif typical of proline directed protein kinases and a web logo extracted from them differentiates from the web logo extracted from all the proline directed phosphosites quantified during our analysis (1151 altogether). A paradoxical outcome of our study was the detection of 116 phosphosites (belonging to 92 proteins altogether) whose occupancy is substantially increased (50% or more), rather than decreased by QZ treatment: 40% of these display the typical motif recognized by proline directed kinases, while about 25% fulfill the CK2 consensus. Collectively taken our data on one side have led to the disclosure of a subset of CK2 targets which are likely to be implicated in the early steps of CK2 signaling counteracting apoptosis, on the other they provide evidence for the existence of side and off-target effects of the CK2 inhibitor quinalizarin, paving the road toward the detection of other kinases susceptible to this compound. This article is part of a Special Issue entitled: Medical Proteomics.

1. Introduction

The acronym CK2 (derived from the misnomer “casein kinase-2” [1]) denotes a constitutively active, very pleiotropic Ser/Thr protein kinase, responsible alone for the generation of a substantial proportion of the phosphoproteome [2] whose abnormally elevated level is often exploited by cancer cells to escape apoptosis [3] and maintain their malignant phenotype [4]. Constitutive activity and pleiotropy, which likely are the two sides of the same coin, reflect the implication of CK2 in a huge variety of cellular functions, a circumstance which makes the dissection of individual signaling pathways affected by CK2 a very complicated job. In order to address this problem a number of cell

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permeable inhibitors have been developed, belonging to several different chemical classes and displaying *in vitro* variable selectivity [5]. A common denominator of these compounds is the ability to induce apoptosis, a property more evident with cancer cells than it is with normal cells, consistent with the concept that many tumor cells rely on high CK2 level for survival [4], a phenomenon also referred to as "non-oncogene addiction" [6]. The cytotoxic efficacy of CK2 inhibitors is invariably paralleled by blockage of endogenous CK2, as judged from the monitoring of its activity in cell lysates [4] being also accompanied by alterations in cell functionality and morphology which are variable depending on the cell lines. However, the actual contribution of CK2 to individual cellular effects observed upon treatment with inhibitors is challenged by a number of arguments, the main one being the lack of absolute selectivity of all available cell permeable CK2 inhibitors, especially at conditions used for in cell experimentation: off-target effects of CK2 inhibitors in fact not only are quite expectable based on their selectivity profiles *in vitro*, but also have been amply documented by functional proteomics analyses [7,8] and shown to apply also to CX-4945 [9], a CK2 inhibitor previously shown to be highly selective and cell permeable [10]. Another complication arising during in cell studies with CK2 inhibitors comes from the variable turnover of the countless phosphosites generated by this kinase. On the average these phosphates turn over very slowly as compared to those incorporated by other kinases, as also outlined by a quantitative MS analysis performed in yeast [11]. On the other hand we have to assume that a subset of CK2 sites turn over much more rapidly than others, since, e.g., 6 h treatment of CEM cells with 0.5 μM CX-4945 (a condition fully abrogating endogenous CK2 activity) is sufficient to promote the almost complete dephosphorylation of Akt pS129, whereas under the same conditions cdc37 pS13, another phosphoresidue generated by CK2, remains nearly unaffected for more than 24 h [12]. Since in the same experiment 6 h treatment with 0.5 μM CX-4945 is sufficient to reduce by 50% cell viability, it is quite conceivable that a rapidly turning over subset of CK2 targets (behaving like Akt pS129 rather than like cdc37 pS13) mainly accounts for the pro-survival/anti-apoptotic role of CK2.

To shed light on this intricate scenario we have performed a quantitative phosphoproteomic study exploiting HEK-293T cells either treated or not with one of the most selective CK2 inhibitors, quinalizarin (QZ) under conditions where endogenous CK2 activity is nearly abolished [13] while the pro-apoptotic efficacy of QZ is not yet evident. The rationale underlying this strategy is that the identification of phosphosites promptly affected by QZ may provide critical information on one side about CK2 targets implicated in the early steps leading to pro-survival signaling, on the other on off-target and side effects of this CK2 inhibitor.

The outcome of this study is described and discussed in this report.

2. Materials and methods

2.1. Materials

Recombinant human RanBP1 and PDCD4 were purchased from ProSpec (Tany TechnoGene Ltd.). All solvents and chemicals were purchased from Sigma and were of MS grade or equivalent.

2.2. Cell viability assay

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide) method. Human Embryonic Kidney (HEK) 293T cells plated in 96-well flat bottom plates (3×10^4 cells/100 μl) were incubated for variable times in the presence of 50 μM quinalizarin or drug vehicle (dimethyl sulfoxide DMSO) as control. 1 h before the end of the incubation, 10 μl of MTT solution (5 mg/ml in Phosphate Buffered Saline PBS, Sigma) was added to each well. Incubations were stopped by addition of 20 μl of lysis solution (20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2% (v/v) acetic acid and 25 mM HCl). Plates were read for OD at λ 590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories). Data reported in Fig. S1 (supplementary material) were obtained from five independent measurements for each condition.

2.3. Cell culture and stable isotope labeling

HEK-293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing either L-$^{13}C_6$-arginine and L-$^{13}C_6$-lysine (Cambridge Isotope Laboratories) or conventional L-arginine and L-lysine (heavy and light medium respectively), supplemented with 1 mM L-glutamine, 1% penicillin/streptomycin, and 10% dialyzed Foetal Bovine Serum (FBS, Invitrogen). The growth of the cells maintained in the heavy medium was not different from the growth in normal (light) medium, as shown by cell morphology and doubling time. Cells were maintained in culture for more than 10 cell doublings before the treatment with the inhibitor was performed.

2.4. Cell treatment with quinalizarin and protein extraction

6 cell cultures (3 heavy and 3 light) were treated for 3 h with 50 μM quinalizarin (QZ) dissolved in DMSO, while 6 samples (3 heavy and 3 light) were kept as control and treated with the same volume of DMSO without inhibitor. After the treatment cells were washed twice with PBS and once with 5 mM Tris–HCl, 250 mM sucrose, pH 7.5. Cells were harvested using scrapers and 500 μl of lysis buffer containing 10 mM Tris–HCl pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, complete protease inhibitor EDTA-free (Roche), and phosphatase inhibitor cocktail (Roche).

Cells were transferred into 2 ml tubes, sonicated 3 times and centrifuged for 15 min at 14,000 ×g. The insoluble pellet was discarded and the supernatant was subjected to ultracentrifugation at 100,000 ×g for 1 h at 4 °C. The pellet (membrane rich fraction) was dissolved in 100 μl of lysis buffer containing 50 mM Tris–HCl pH 7.5, 1% SDS, complete proteases inhibitors EDTA-free (Roche), and phosphatase inhibitor cocktail (Roche). Finally protein extracts (soluble and membrane rich fractions) were pooled together and carefully quantified using the Micro-Lowry Peterson's Modification Total Protein Kit (Sigma).

2.5. SDS-PAGE and in-gel protein digestion

100 μg of proteins from heavy cells were mixed with 100 μg of proteins from light cells to obtain 6 independent replicates (3 "forward" experiments and 3 "reverse" experiments), as outlined in Fig. 1. Samples were loaded onto a 4–12% SDS-PAGE precast gels (NuPAGE Bis-Tris Gel, Invitrogen), and proteins separated at 200 V for 1 h. Gels were stained with SimplyBlue coomassie (Invitrogen) and destained overnight in water. Each gel lane was subjected to in-gel protein digestion as described in [14]. Finally, 200 μg of extracted peptides for each experimental replicate was dried under vacuum and kept at −80 °C until phosphopeptide enrichment was performed.

2.6. Phosphopeptide enrichment

Samples were dissolved in 60 μl of 80% acetonitrile (ACN), 6% trifluoroacetic acid (TFA) (loading/washing buffer). Phosphopeptide enrichment was performed with homemade micro-columns prepared by inserting 1.2 mg of TiO_2 (Titansphere, GL Sciences Inc.) into Stage Tips (C18 material, Proxeon). Columns were conditioned twice (50 μl) with ACN and twice (50 μl) with loading buffer. Samples were slowly loaded into the micro-columns, and the resin was washed four times (50 μl each time) with washing buffer and twice with 0.1% TFA (50 μl each time). Retained peptides were eluted with 50 μl of 5% ammonium hydroxide freshly prepared (pH ≈ 11.0). A last passage of elution from the C18 filter was performed with 50% ACN, 0.1% formic acid (FA) and samples were acidified with FA, dried under vacuum and suspended in 0.1% FA for LC–MS/MS analysis.

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