en TiO2. El análisis de la fracción retenida en TiO2, con el motor Spectrum Mill y la base de datos humana IPI, permitió la identificación de 116 fosfopéptidos. En contraste, sólo 11 fosfopéptidos se identificaron cuando la misma muestra se analizó con el HPLC-Chip estándar. Como era de esperar, los componentes mayoritarios no fosforilados, enmascararon la identificación de los fosfopéptidos en muestras en las que no se realizó el paso de enriquecimiento/ separación en TiO2. Ello es debido a la falta de tiempo para adquirir en un pico cromatográfico, los espectros de MS/MS de péptidos minoritarios (como los fosforilados), cuando estos coeluyen con diversos péptidos mayoritarios y se trabaja en Auto-MS/MS.

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Identification of a new phosphorylation site in E2F1 transcription factor

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The E2F transcription factor family (E2F1-8) is required for the regulation of a number of genes involved in several essential cellular processes. It is for that reason that the activity of E2Fs must be carefully controlled. Reversible phosphorylation is one of the main regulatory mechanisms controlling the activity of proteins that has also been described for E2Fs. Several phosphorylation events have already been described for E2F1 but the ones described so far are insufficient to completely understand how this transcription factor is regulated. In order to identify new phosphorylation sites, E2F1 was immunoprecipitated and following protease mediated digestion was loaded onto TiO_2 microcolumns. The enriched fractions were analyzed by LC-MS/MS. This methodology allowed the identification of a new E2F1 phosphorylation site not described to date.

Introduction

E2Fs (E2F1-8) play a central role in cell cycle progression, DNA damage repair, apoptosis, cell differentiation and development. Each of this family members are known to regulate more than one cellular process depending on the stimuli they are subjected to. For instance, E2F1-dependent transcription is essential for cell proliferation but depending on the cellular context can also trigger cell death. That is why E2F-dependent transcription needs to be tightly regulated. Transcriptional activity of each E2F is modulated at various levels. Not only protein-protein interaction and subcellular localization but also post-translational modifications such as acetylation and phosphorylation have been proven to regulate and determine the specific roles of E2Fs. For instance, it is known that the DNA binding capacity

of E2F1 is reduced when Ser375 is phosphorylated [1] while phosphorylation of Ser31 and Ser364 following DNA damage has been reported to stabilize E2F1 itself and also promote its apoptotic pathway [2]. However it cannot be ruled out the possibility to find more phosphorylation events in E2F1.

To gain a better understanding of how E2F1 is regulated, we looked for new phosphorylation events it may suffer. Immunoprecipitation of E2F1 followed by LC-MS/MS analysis of TiO_2 enriched phosphopeptides (Figure 1.) led to the identification of a novel phosphorylation site for E2F1.

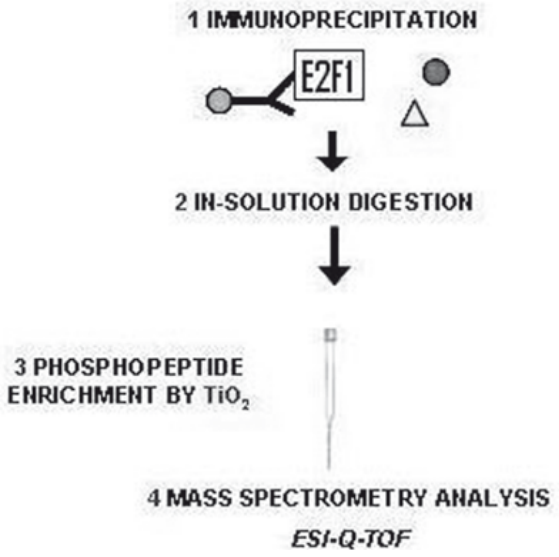

Figure 1. *Strategy for the analysis of phosphorylation in E2F1. E2F1 was immunoprecipitated, protease-digested and the resulting peptides were enriched by TiO_2 and analyzed by LC-MS/MS.*

Materials and methods

Immunoprecipitation of E2F1 transcription factor: A cell extract from 293T cells overexpressing E2F1 was incubated with an antibody against E2F1. After washing, proteins were eluted with 0.1M glycine pH 2.5 at 25ºC O/N. Samples were neutralized with 1.5M TrisHCl pH 8.8. *In-solution digestion of the immnoprecipitated fractions*: Protein eluates were reduced with dithiothreitol, alkylated with iodoacetamide and digested O/N at 25ºC with GluC. *Titanium Dioxide (TiO2) enrichment of phosphorylated peptides*: Preparation and use of TiO_2 microcolumns for phosphopeptide enrichment was performed essentially as previously described by Larsen et al., 2005 [3]. *LC-MS/MS*: Titanium enriched fractions were analyzed in a SYNAPT HDMS mass spectrometer (Waters) interfaced to a NanoAcquity UPLC System (Waters). The sample was loaded onto a Symmetry 300 C18 precolumn. The precolumn was connected to a BEH130 C18 column (Waters). Mascot search engine (http://www.matrixscience.com) was used for protein identification. *Bioinformatics*: Phosphorylation site- and motif-prediction tools were used. Netphos 2.0 [4] and Phosida [5] were used for the prediction of putative phosphorylation sites in E2F1. Additionally, Phosida was used for kinase prediction of putative phosphorylation sites. http://www.cbs.dtu.dk/services/NetPhos/ and http://www.phosida.com/

Results and discussion

A new phosphorylation site was identified for E2F1. Manual validation of the spectra allowed the assignment of the phosphorylation to residue Ser307 (Figure 2).

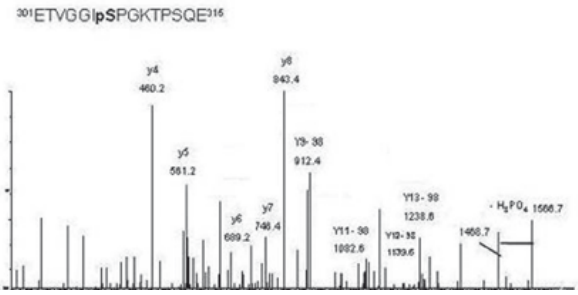

Figure 2. *MS/MS spectra of the identified phosphopeptide for E2F1 transcription factor. Among the four possible phosphorylable residues for the phosphopeptide ^{301}ETVGGIS-PGKTPSQE^{315}, it was possible to assign the phosphorylation site to residue Ser307.*

Bioinformatic analysis was also performed to predict possible phosphorylation sites in E2F1. According to NetPhos 2.0 predictor, the phosphorylation predicted score for the identified site in E2F1 is 0.997, which reinforces our result. Moreover, phosphorylation database Phosida predicted that the kinase involved in the phosphorylation of Ser307 would be CDK2. It has already been described that E2F1 can form a stable complex with cyclinA/CDK2 and that CDK2 can phosphorylate E2F1 regulating this way its DNA-binding activity [1].

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Establecimiento de un flujo de trabajo efectivo en la caracterización cualitativa y cuantitativa del fosfoproteoma

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La determinación de puntos de fosforilación en proteínas es una de las áreas de la proteómica con mayor interés en la actualidad. Los últimos avances en la instrumentación y en las técnicas de purificación están permitiendo la detección de formas fosforiladas de péptidos a muy bajas concentraciones y con gran fiabilidad. La caracterización del denominado fosfoproteoma hace posible la obtención de instantáneas del estado de fosforilación de un determinado organismo y su cuantificación en diferentes estados. No obstante, debido a las utilización de métodos de enriquecimiento y a los barridos específicos utilizados en el análisis por espectrometría de masas, el análisis de los datos generados aumenta sensiblemente en complejidad respecto a otros análisis proteómicos clásicos. Es por tanto necesario el establecimiento de un flujo de trabajo específico para fosfoproteómica con objeto de responder a las diversas necesidades que las técnicas involucradas requieren.

1. Peculiaridades de los datos obtenidos en la identificación de fosfopéptidos

En el analisis de fosfopéptidos mediante técnicas de *shotgun proteomics* deben tenerse en cuenta las siguientes consideraciones:

- La evaluación de la confianza en la identificación de fosfoproteínas en base al número de péptidos identificados no siempre es posible dado que en las etapas de enriquecimiento gran parte de los péptidos no fosforilados se eliminan. La información analítica reportada debe por tanto tener en cuenta todas las proteínas a las que apuntan estos péptidos únicos, tanto a efectos cualitativos como cuantitativos.

- El número de modificaciones a tener en cuenta durante la identificación de los espectros de fragmentación (al menos 3 modificaciones variables para S/T/Y en MS^2, y dos más para S/T en MS^3) y las características espectrales de los fosfopéptidos (pérdida preponderante del grupo fosfato) facilitan la aparición de falsos positivos. La utilización de bases de datos señuelo nos permite obtener una estimación de la proporcion de falsos positivos en el conjunto de fosfopéptidos validados *(FDR o False Discovery Rate)* [4, 5].

- Un elemento clave en la asignación de puntos de fosforilación es la correcta identificación del aminoácido modificado cuando exista más de una alternativa posible. En estos casos, los buscadores en base de datos como SEQUEST,