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Addition/Correction

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Correction to Feasibility of Large-Scale Phosphoproteomics with Higher Energy Collisional Dissociation Fragmentation

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The large-scale analysis of phosphorylation sites can be performed in many different formats.[1] In a recent publication,[2] we investigated whether the relatively new method of higher energy collisional dissociation (HCD)[3,4] is a viable fragmentation method for phosphoproteomics. This was an important and open question because HCD has certain differences from CID, the method overwhelmingly used on Orbitrap instruments. For comparison purposes, the paper also contains CID experiments using the same samples. When used in a high resolution mode (measurement of the fragments in the Orbitrap analyzer), we found that CID and HCD identified about the same number of phosphopeptides. These data unambiguously established that HCD on the novel Orbitrap Velos platform indeed allows identification of thousands of phosphorylation sites.

In a further analysis, we compared CID with readout at a low resolution in the linear ion trap with HCD with read out in the Orbitrap. Remarkably, in view of the larger number of precursor ions that are thought to be necessary in HCD than in low resolution phospho CID with read out in an ion trap, we found that HCD was not performing worse than CID. We investigated this phenomenon in more detail by performing analyses with fewer and fewer precursor ions, which revealed that the identification percentage was largely unchanged down to precursor ion numbers of only 5000. These experiments suggested that HCD with high resolution Orbitrap readout by its higher data quality makes up for the somewhat slower speed and sensitivity compared to the combination of CID and linear ion trap fragment analysis.

In this comparison, we unfortunately specified the exclusion window for avoiding resequencing for the linear ion trap measurements in Da instead of ppm. (Note that this was not the case in the direct comparison of CID and HCD discussed above; see Table 1 of the manuscript). This could have had the effect of limiting the number of CID spectra compared to HCD spectra and thereby skewing the comparison as has been speculated in ref 5. Shortly after publication of our paper, scientists at Thermo made us aware of this mistake using our publicly deposited data, an advantage of raw data deposition that we have advocated strongly but which is unfortunately still not the norm in proteomics.[6] We then immediately checked if this mistake had any material effect on the results of this particular comparison, but fortunately, this was not the case, which we established by directly comparing CID with ion trap read out and HCD with Orbitrap analysis. In response to the questions raised in ref 5, we additionally acquired new data comparing the HCD and the CID settings for phosphosite mapping in an Orbitrap Velos instrument in a fashion where one HCD measurement was directly followed by one CID measurement to avoid any possible systematic bias. Again, the data clearly shows that results by CID and HCD are comparable (Figure 1), the same finding as in the original paper. Further support for our observation that HCD is a viable method of large-scale phosphoanalysis came from several papers that have been published in the meantime by leading phosphoproteomics laboratories. For instance both the Heck group and the Coon group have compared CID and HCD and reported that HCD outperforms CID for the analysis of phosphopeptides (see Figure 1a in ref 7 and Figure 1 in ref 8) in contrast to the findings in ref 5. In an effort to find a reason why the researchers of ref 5 obtained many more sites by low resolution CID than by high resolution HCD, we checked their HCD settings and found that they were suboptimal. They added a delay to each MS/MS scan by specifying an "activation time" of 30 ms. However in HCD there is no function for this parameter because unlike in an ion trap, ions are not "activated" by RF in HCD, and we routinely set it to 0.1 ms. Furthermore, they used a minimum signal threshold of 500, which is the usual setting for the ion trap; in HCD this threshold is generally set to 5000 or higher. However, we do not believe that these settings would handicap HCD sufficiently to explain its relative underperformance in ref 5.

As a result of its excellent performance, in our laboratory and many others, HCD has become the method of choice for both proteome and phosphoproteome analysis. We have also found that the novel quadrupole Orbitrap instrument, the Q Exactive,[9] which does not offer the possibility of CID at all, is nevertheless extremely powerful at proteome and phosphoproteome analysis. It features even better MS/MS cycle times than low resolution CID. To the extent that higher numbers in low resolution CID experiments in some researchers' hands was simply due to the speed of MS/MS acquisition, this advantage should now also be matched by HCD.

In our paper, we report identification of 9668 class I phosphorylation sites, with mean localization probability of 0.997, on which all the analysis in the paper is based. We also mention that 16,559 distinct sites were identified altogether. This is the number reported by MaxQuant in the phosphosite text output for the combined experiments, but these are not necessarily localized, although they represent different measurements and potentially different sites on the peptide. These peptides are reported for use by the community because the probability that they are phosphopeptides with the identified sequence is greater than 99%. However, these class II and III sites are not meant to be used as distinct phosphorylation patterns. This should have been clear because these peptides are each listed with site-specific localization probabilities, and if this probability is very low, then by definition the

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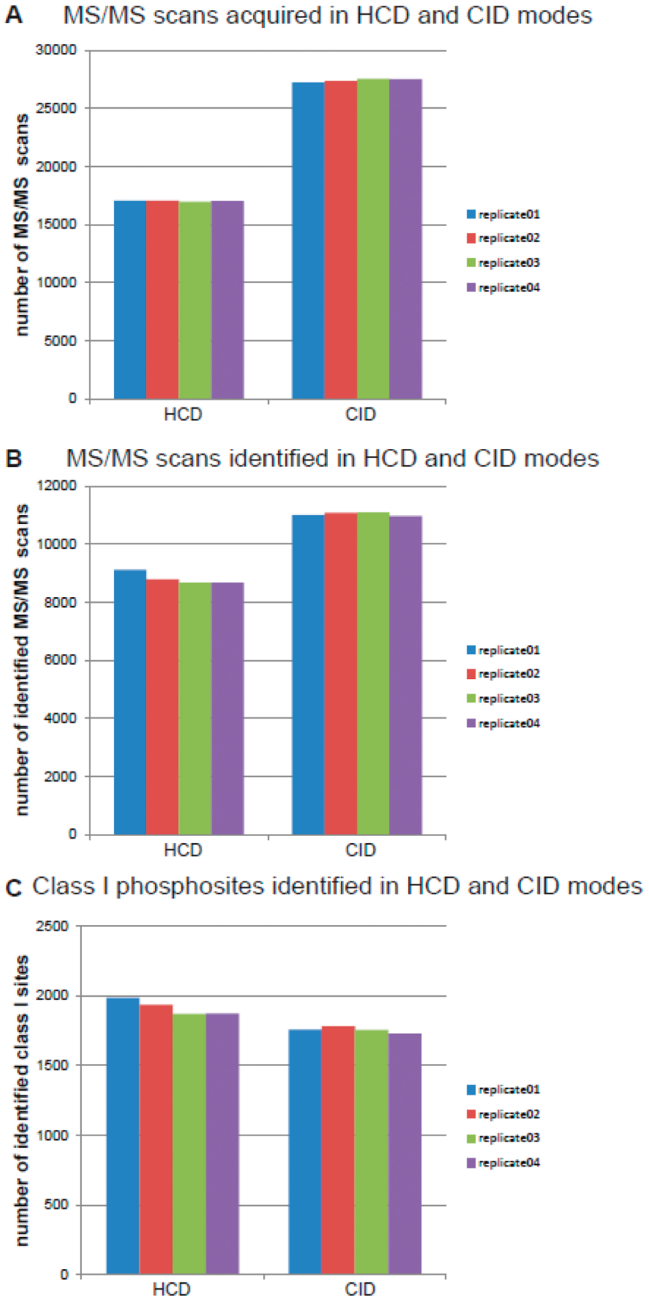

Figure 1. Performance of CID and HCD methods on LTQ Orbitrap Velos. (A) Number of performed MS2 scans in CID and HCD method settings. The number of identified MS2 scans and the Class 1 phosphosites are shown in panels B and C, respectively.

phosphogroup could have been at another site. (This is the very reason we distinguish class I sites from other sites.) We nevertheless report them because they may be useful for follow up by researchers interested in particular proteins. In any case this has no bearing on the comparison of CID and HCD at all, which is based only on class I sites.

In summary, none of the conclusions of the paper are impacted, and we note that the central message, i.e., that HCD is a powerful new member of the phosphoproteomics analysis toolbox in addition to the existing, proven fragmentation modes like ion trap CID and ETD, has been validated by several laboratories in addition to our own as well as implicitly by the above-mentioned introduction of the Q Exactive, which exclusively has the HCD fragmentation option. The setting of Da instead of ppm in the dynamic exclusion window did not change the conclusion that the HCD platform can perform as well as the CID platform in any way. That said, we believe that CID remains a very important fragmentation method, especially if coupled with fragment measurement at high resolution. For instance, ion traps readily allow MS/MS^n experiments, something that is in principle but currently not routinely possible using HCD.[10] Furthermore, recent work on the LTQ-Orbitrap Elite clearly shows that it is now feasible to combine high resolution CID and HCD spectra in the same cycle time that was previously necessary to perform one fragmentation method alone.[11] In conclusion, we believe that CID, ETD, and HCD are all powerful methods for phosphopeptide analysis, especially when used at high resolution, and that the proteomics field can only gain by adding a new method to its tool-box.

EXPERIMENTAL PROCEDURES

Sample Preparation and LC–MS Analysis

Phosphopeptides were enriched from total HeLa peptides using titanium affinity as described earlier.[2] Briefly, the tryptic peptides were incubated with titanium dioxide beads and dihydro benzoic acid in the presence of 30% acetonitrile and 0.1% trifluoroacetic acid for 30 min. The phosphopeptide bound beads were then washed to remove nonphosphopeptides and eluted with ammonia. The enriched phosphopeptides were then desalted on C18 Empore disk StageTips, eluted with 40% acetonitrile, and concentrated to remove organic solvents.

Peptides were separated in a 15 cm column (75 μm inner diameter) packed in-house with 3 μm C_{18} beads (Reprosil-AQ Pur, Dr. Maisch) on a Proxeon EASY-nLC system (Proxeon Biosystems, Odense, Denmark, now Thermo Fisher Scientific) using a binary gradient of 138 min using buffer A (0.5% acetic acid) and buffer B (0.5% acetic acid and 80% ACN) coupled online to an LTQ Orbitrap Velos instrument (Thermo Fisher Scientific). For the high–high strategy, survey scans were acquired in the Orbitrap mass analyzer with resolution 30,000 at m/z 400. For the full scans, 1E6 ions were accumulated in the C trap (injection time limited to a maximum of 250 ms) and detected in the Orbitrap analyzer. The 10 most intense ions with charge states $\geq$2 were sequentially isolated using a 4 Th isolation window (signal threshold of 10,000) to a target value of 3E4 with a maximum injection time of 150 ms and fragmented by HCD in the collision cell (normalized collision energy of 40%) and detected in the Orbitrap analyzer at 7,500 resolution. For the high–low strategy using CID, full scans were acquired in the Orbitrap analyzer at 60,000 resolution because parallel acquisition is enabled in the high-low mode. Up to the 20 most intense peaks with charge state $\geq$2 were selected for sequencing (signal threshold of 1000) to a target value of 5,000 with a maximum injection time of 25 ms, isolation window of 2 Th and fragmented in the ion trap by collision induced dissociation with normalized collision energy of 35%, activation q = 0.25, and activation time of 10 ms. For CID "wideband activation" and "multistage activation" options were enabled with the appropriate neutral loss mass list for singly, doubly, and triply phosphorylated peptides. The fragmentation spectra were acquired in the ion trap at normal scan rate by lateral ejection and recorded by the dynode-multiplier system. These parameters are the same as in our previous publication with the exception of the dynamic

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exclusion window, which was set to 5 to the low mass side of the peak and 10 ppm to its high mass side for both CID and HCD methods (this is the parameter that we set incorrectly before). To avoid any systematic bias the instrument was alternated between the two methods while performing quadruplicate measurements.

Data analysis

The data were analyzed with MaxQuant[12] version 1.2.6.7 and processed according to the standard workflow. MaxQuant now also incorporates Andromeda,[13] a probabilistic search engine. We used the human IPI database version 3.68 with initial precursor mass tolerance of 7 ppm and an allowed fragment mass deviation of 20 ppm for the HCD strategy. For the CID these values were 7 ppm and 0.5 Th, respectively. The search included cysteine carbamidomethylation as a fixed modification and N-acetylation of protein, oxidation of methionine, and phosphorylation of STY as variable modifications. Up to two missed cleavages were allowed for protease digestion, and a minimum of 6 amino acids were required for valid peptide identifications.

Data Availability

The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash: jo9kGl0s7ZzC7mHw7yUNerlmoe0kfUQpJhOqQnXyc5o14on2PQ7yLQJ/z+MrqODgRGFnXboDL1NUfh95tpsVP6gAzfAAAAAAAAAISA== Passphrase: cuix0oZyhl0riLbzTmgx

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