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Review

Connexin multi-site phosphorylation: Mass spectrometry-based proteomics fills the gap ☆

Vincent C. Chen [a,b,*], Joost W. Gouw [a], Christian C. Naus [b,1,2], Leonard J. Foster [a,1,3]

[a] Department of Biochemistry and Molecular Biology, Centre for High-Throughput Biology, University of British Columbia, 2125 East Mall, Vancouver, British Columbia, Canada V6T 1Z4
[b] Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3

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ABSTRACT

Connexins require an integrated network for protein synthesis, assembly, gating, internalization, degradation and feedback control that are necessary to regulate the biosynthesis, and turnover of gap junction channels. At the most fundamental level, the introduction of sequence-altering, modifications introduces changes in protein conformation, activity, charge, stability and localization. Understanding the sites, patterns and magnitude of protein post-translational modification, including phosphorylation, is absolutely critical. Historically, the examination of connexin phosphorylation has been placed within the context that one or small number of sites of modification strictly corresponds to one molecular function. However, the release of high-profile proteomic datasets appears to challenge this dogma by demonstrating connexins undergo multiple levels of multi-site phosphorylation. With the growing prominence of mass spectrometry in biology and medicine, we are now getting a glimpse of the richness of connexin phosphate signals. Having implications to health and disease, this review provides an overview of technologies in the context of targeted and discovery proteomics, and further discusses how these techniques are being applied to "fill the gaps" in understanding of connexin post-translational control. This article is part of a Special Issue entitled: The Communicating junctions, roles and dysfunctions.

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1. Introduction

The connexin (Cx) family of proteins are required for a range of biological processes including embryonic development [1,2], homeostasis [3,4], calcium signaling [5], cell migration [2,6] and apoptosis [7]. As a tetraspan-integral membrane protein, resident of the secretory pathway and principal component of gap junctions (GJs), Cx require an integrated network for protein synthesis, transport, assembly, gating, internalization, degradation and feedback control, with each stage likely to be critical in the control of GJ intercellular communication. As

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* Corresponding author at: Department of Biochemistry and Molecular Biology, Centre for High-Throughput Biology, University of British Columbia, 2125 East Mall, Vancouver, British Columbia, Canada V6T 1Z4. Tel.: +1 604 827 3436.
E-mail addresses: vincent.chen@ubc.ca (V.C. Chen), cnaus@exchange.ubc.ca (C.C. Naus), Foster@chibi.ca (L.J. Foster).
[1] Co-senior authorship.
[2] Tel.: +1 604 827 4383; fax: +1 604 822 2316.
[3] Tel.: +1 604 822 8311; fax: +1 604 822 5227.

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an especially dynamic component of the proteome, the site-specific phosphorylation/dephosphorylation is thought to occur as Cx subunits are trafficked from one cellular compartment to the other [8–12]. The life-span of most Cxs is incredibly short, and Fig. 1 gives a simplified, but a generally accepted summary related to the "timing" of GJ subunit assembly and turnover. This review starts by discussing how mass spectrometry (MS)-based proteomics may be used to "fill the gaps" in our understanding of GJ/Cx regulation. The specific roles of Cx phosphorylation have been recently discussed [9,10,13], and the purpose of this review is to introduce how proteomics may be applied to better understand the functions, sites, magnitude and sequence of multi-site phosphorylation that encompass the 'Cx code'.

The molecular heterogeneity of a protein's post-translational modification (PTM) is influenced by, and dependent upon, an interplay of enzymes, physiological conditions and the cell-/tissue-type. At the most fundamental level, the introduction of sequence-altering modifications, either dynamic (e.g. phosphorylation, ubiquitylation) or non-reversible (e.g., cleavage, degradation), introduces changes in protein conformation, activity, charge, stability and localization. Furthermore, with more than ten different phosphorylation-specific protein-binding domains [14], the addition of phosphate to one or multiple amino acid residues often modulates docking sites for protein–protein interactions. By SDS-PAGE, Cx43 typically forms multiple bands between ~37 and ~45 kDa, with the fastest migrating species recognized as non-phosphorylated protein. In the early stages of Cx43 assembly the protein is largely non-phosphorylated [11], with extensive modification occurring only after transport to the plasma membrane [15]. Despite the wealth of information related to Cx phosphorylation, our current body of knowledge related to Cx PTMs and their impact on function is often viewed as incomplete, and at times, contradictory [16]. Although monitoring the state of phosphorylation by SDS-PAGE has proven to be useful for Cx43, it should be noted not all phosphoproteins demonstrate detectable electrophoretic shifts, and the identity of individual site(s) and modifying enzyme(s) largely remain unresolved. Driven by the hypothesis that one or a small set of individual sites correspond to one molecular function, the determination of phospho-sites historically required the use of P^{32} radiolabels/2D gels, phospho-specific antibodies, nullifying site-directed conversion of Ser/Thr to Ala or Tyr to Phe, and/or phospho-mimetic mutations to Asp or Glu. The precise role of individual sites of modification alone or in combination within specific subcellular locales has been difficult to pinpoint because of the lack of side chain analogs that allow one to investigate functional contributions of phosphorylation alone. For example, it important to consider that substitution of Ser/Thr/Try to Ala or Phe not only lacks the ability to accept phosphate, but also increases hydrophobicity by displacing the position's ability to accept hydrogen bonds with water. With varying degrees of success [17–21], discussions regarding Glu/Asp phospho-mimetics largely center on obvious differences in the number of oxygen atoms/hydrogen bonds, geometry, numbers of negative charge at neutral pH, size and the lack of a natural pTyr isostere [22]. While individual sites of phosphorylation may be sufficient to induce an on/off response, compounding these challenges PTMs have also recently been found to work in a cooperative manner [23,24]. Best exemplified by gene regulation and the histone code, core histones are reversibly modulated by synergistic phosphorylation, acetylation, methylation and ubiquitylation [25,26]. With similar hypotheses having also been put forward for p53 [27] and tubulin [28], it is now clear multi-site PTMs represent an important mechanism for cell signal transduction. For Cx43 sequence harboring 66 Ser/Thr/Tyr residues, with the majority of confirmed sites residing in the cytoplasmic carboxyl-terminal tail (CT), it is important to recognize that a single Cx43 subunit may encompass upwards of 10^92 (66!) possible phosphate-based signaling combinations. It is clear the consequences of multi-site phosphorylation with respect to Cx trafficking/life-cycle and GJ gating have yet to be fully appreciated.

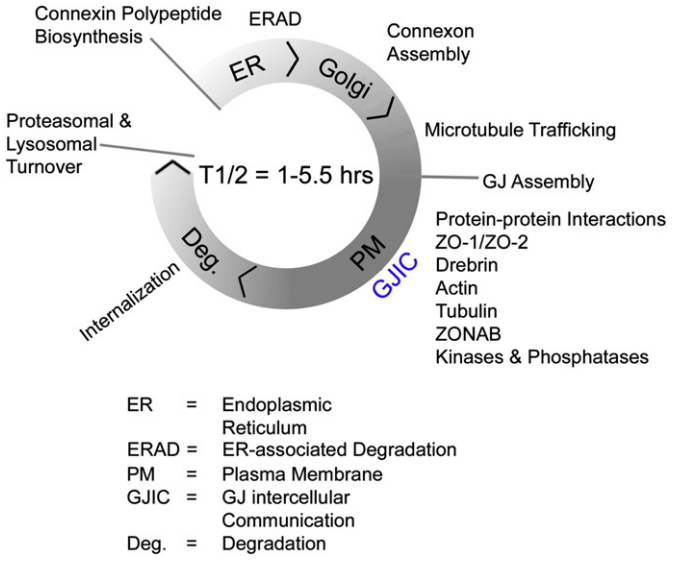

Fig. 1. Overview of the GJ life-cycle, depicting the relative order and timing of Cx subunits. Newly synthesized Cxs undergo synthesis within the ER undergo quality control within the ER-associated degradation pathway (ERAD). Subunits passing this stage undergo vesicular transport to the Golgi where they are oligomerized into heximeric "connexons". From here, connexon enriched vesicles are delivered to the plasma membrane where they dock at sites of cell-to-cell contact to establish or maintain sites of GJ intercellular communication (GJIC). While at GJs, Cxs-bind to an array of proteins, including cytoskeletal proteins (ZO-1, ZO-2, MUPP1, actin, drebrin, tubulin), transcription factors (ZONAB) and a variety of signaling kinases and phosphatases that are thought scaffold channels and potentiate GJ communication levels. A mechanism of GJ degradation involves internalization as double membrane structures called annular GJs that interface with the lysosome and/or proteasome. Most Cxs examined thus far demonstrate a half-life on the order of 1–5.5 h. Such rates of production and turnover, alongside complex patterns of assembly and trafficking, are consistent with a highly integrated system.

2. Proteomics

In its most classical definition, a proteome is defined as the set of proteins expressed by a genome [29,30]. While omic-based approaches to address specific research questions are often described as "fishing experiments", these strategies have their merits as they provide an unbiased view of biology that could otherwise never have been predicted. Proteomics technology is improving rapidly and now endeavors to systematically examine protein expression, structure, function, protein–protein interaction and dynamics. The proteome is highly dynamic, with a broad range of individual protein concentrations ranging from several million to only a few copies/cell [31]. Although sub-stoichiometric phosphorylation may be sufficient to induce a robust biological signal [32,33], the detection of discrete changes in protein phosphorylation amongst the bulk proteome are not trivial. In general, to identify a protein by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), the instrument should be able to (i) determine the peptide's mass, (ii) fragment the peptide and analyze fragment mass, and in some instances, (iii) quantify expression or level of biochemical enrichment (Fig. 2). The lack of time for stages i and ii due to the complexity of digests makes sample preparation an important feature of most proteomic endeavors. An ongoing theme in the field is to therefore increase analytical sensitivity, by increasing instrumental speed and/or fractionation of the proteome by chemical, biochemical and genetic methods [34–36].

3. Mass spectrometry (MS)

The first step in proteomic analysis involves reducing proteins to peptides with an endoproteinase, such as trypsin. Cleaving carboxy-