

You can Never have too many kinases: The Sodium Hydrogen Exchanger Isoform 1 Regulation by Phosphorylation

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Abstract

Regulation of Na⁺-H⁺ exchanger isoform 1 (NHE1) activity is a dynamic, integrated system demonstrating the complexity of kinase signaling. Studies of NHE1 have described 22 confirmed and putative phosphorylation sites on the cytoplasmic domain regulated by 12 protein kinases. However the final number of sites and the impact of these sites remains unclear. By identifying the Rock phosphorylation site and its role in regulating NHE1 activity, we began to understand the functional interplay between the RhoA/Rock and Rsk/Erk pathways. This demonstrates the need to have a complete and comprehensive understanding of the relationship between each kinase involved in NHE1 modification for a true understanding of the exchanger's role in the control of cellular biological activity. By observing how the Rock phosphorylation site impacts cellular proliferation, it was demonstrated that Rock activity is only partially responsible for NHE1's impact on biological activity. Furthermore, data presented here shows that Rock activity is involved in both α 1-adrenergic (Phenylephrine) and LPA signaling but not PDGF activation of NHE1 transport activity suggesting that growth factor signaling does not require Rock, yet the kinase is essential in the effects of GPCR pathways. Of particular interest and adding to the complexity of kinase signaling, Pyk2 activity has been found to have an inhibitory effect on NHE1. These data demonstrate an uncertainty in the exact role that different kinases play in the regulation of NHE1 function, yet succeeds in providing evidence of the intricacy of phosphorylation organization and a need for further elucidation of the interplay between phosphorylation sites. Overall, the pivotal role of NHE1 in disease establishment and progression creates a solid argument for continued efforts in understanding its regulation.

Keywords: NHE1; Sodium hydrogen exchanger isoform 1; Protein kinase; ERK; Rsk; Rock; Phosphorylation

Introduction

The Na⁺-H⁺ exchanger isoform 1 (NHE1; SLC9A1) is ubiquitously expressed in human cells where it is a major regulator of intracellular pH (pHi) and cell volume (for reviews see [1-3]). Alterations in NHE1 activity are essential in the regulation of cell proliferation, migration, and survival. NHE1's role in the regulation of cell proliferation is multifaceted. Activation of NHE1 induces an alkalization of pHi permissive for cell growth and passage through both the G1 checkpoint and the G2/M transition of the cell cycle. Additionally, this alkalization promotes cell survival by limiting apoptosis. As a key regulator of cell migration, NHE1 distributes to the leading edge of migrating cells where it serves as a point of attachment for the actin cytoskeleton with the plasma membrane. NHE1 activation is not only essential for normal physiological function; it also plays a central role in pathophysiological activation of cancer and cardiovascular disease.

Neoplastic transformation activation is supported by NHE1 mediated-increase in cell proliferation and the development of unique tumor microenvironment that promotes genetic instability and enhances invasion and metastasis. The acidification of the extracellular environment by NHE1 in developing tumors, induces the activation of extracellular proteases (MMP-2, MMP-9 [4,5]) in regions immediately adjacent to the formation of lamellipodia and invadopodia supporting

localized invasion that ultimately leads to cancer metastasis. The expression level and activity of NHE1 are elevated in a variety of cardiovascular disease states including ischemia/reperfusion injury, cardiac hypertrophy, hypertension, and diabetic cardiomyopathy [3].

During ischemic events, cardiac myocytes switch to anaerobic metabolism resulting in accumulation of lactic acid. The accompanying decrease in pHi stimulates NHE1 activity inducing an accumulation of intracellular Na⁺. The build-up of Na⁺ leads to a reversal of the Na⁺/Ca²⁺ exchanger driving the accumulation of Ca²⁺ that ultimately drive cell death [6].

NHE1 is a 12-pass transmembrane transport protein consisting of 815 amino acids. The N-terminal transport domain resides in the plasma membrane and exchanges a single extracellular sodium ion for one intracellular hydrogen ion. This domain includes a proton sensor region that establishes the set-point for pHi. NHE1 also has an extended C-terminal cytoplasmic tail that function as the transporters regulatory domain. The cytoplasmic regulatory domain includes an extensive series of binding motifs for proteins and lipids as well as numerous phosphorylation sites that have been characterized or proposed (Figure 1). NHE1 transport activity can be increased either through the binding of cofactor proteins or phosphorylation. Additionally, the C-terminus of NHE1 functions as a docking location for the assembly of protein signaling complexes and provides an anchor point between cytoskeletal proteins and the plasma membrane.

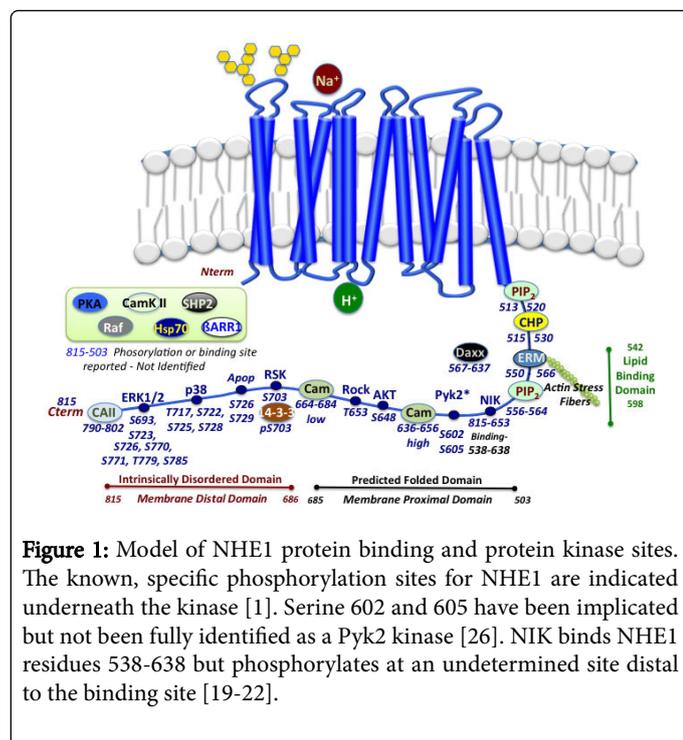


Figure 1: Model of NHE1 protein binding and protein kinase sites. The known, specific phosphorylation sites for NHE1 are indicated underneath the kinase [1]. Serine 602 and 605 have been implicated but not been fully identified as a Pyk2 kinase [26]. NIK binds NHE1 residues 538-638 but phosphorylates at an undetermined site distal to the binding site [19-22].

Recently, we identified threonine 653 (T653) as the RhoA Kinase (Rock) phosphorylation site on NHE1 [7]. That publication was an extension of an earlier report [8] where we demonstrated that both Rock and p90Rsk regulated NHE1 activity and cellular function following stimulation by lysophosphatidic acid (LPA) in Chinese hamster lung fibroblasts. Prior to that time, the p90Rsk phosphorylation site had been identified [9,10] and phosphorylation of NHE1 by Rock had been demonstrated [11] but the synergistic role of the Erk growth factor pathway and RhoA/Rock signaling pathway in the regulation of NHE1 had not been recognized. Identification of the Rock phosphorylation site allowed us to systematically evaluate the role of these two phosphorylation sites on NHE1 transport activity, cytoskeletal organization and cell migration.

The essential findings of this work were that the Rock and Rsk phosphorylation sites displayed additivity in their impact on NHE1 transport activity, where both contributed to intracellular pH change and the pHi set-point. In contrast we found that both phosphorylation sites were found to be critical in regulating stress fiber formation and cell migration. As described here, this work agreed with some but not all of our understanding of ERK, Rsk and Rock signaling. These data indicated a clear distinction between the regulation of NHE1 transport activity and the downstream effectors of biological function related to NHE1, further illuminating the potential complexity of the NHE1 regulation through phosphorylation, and necessitate questioning the mechanistic role of NHE1 multi-phosphorylation. In response, we ask the question, can a protein ever have too many kinases?

NHE1 Phosphorylation

The extended carboxyl terminal of NHE1 (ctNHE) is highly modified with protein and lipid interactions and is reported to be phosphorylated by up to eleven different protein kinases (for a table of

phosphorylation sites and binding proteins, see our review [1]. Five protein kinases (AKT, p38, ERK, Rsk, and Rock) have been reported to phosphorylate at 14 distinct sites. One protein kinase (NIK) has been identified to target NHE1 but the actual residues phosphorylated have not been determined while two other kinases (Pyk2 and Daxx) are also involved in regulating NHE1, possibly directly [1,3]. Phosphorylation has been reported for four NHE1 amino acids (602, 605, 676, 729) but the kinase responsible is not clear. Three additional kinases have been reported to bind and phosphorylate NHE1 but the site identity and functional impact of the modification is not clear. Overall this represents 22 putative and known phosphorylation sites regulated by 12 protein kinases. Most of the phosphorylation sites are in the distal ctNHE domain (Figure 1) lying within the intrinsic disordered domain that may link NHE1 regulation to structural changes induced by phosphorylation [12]. The abundance of phosphorylation sites on NHE1 has led researchers to question the coordination and hierarchy of these sites. However, as the number of sites and their roles are still being identified, no one has systematically dissected the mechanistic impact of each site or expanded these investigations to explore the interplay and continuity of signaling between different pathways. Here we highlight the current understanding and incongruences regarding NHE1 phosphorylation in the literature and outline the importance of developing an integrated model of the mechanism governing NHE1 regulation by phosphorylation.

What make the impact of multiple regulatory sites are the many biological roles NHE1 plays in cell biology/biochemistry. In addition to intra- and extra-cellular pH homeostasis, NHE1 is involved in the regulation of matrix metalloproteinase maturation and activity, actin stress fiber anchoring and formation, and serves as a membrane localization site for several proteins [1-3]. Several investigators show the importance of phosphorylation in regulating these events including our earlier work defining the role of Rsk and Rock on NHE1 phosphorylation [7]. Thus learning how these kinases collaborate or the hierarchy of phosphorylation will help our understanding of these events.

ERK Signaling

G Protein-coupled Receptor (GPCR) and growth factor receptor signaling demonstrate the diversity in protein kinase regulation of NHE1. Both receptor family pathways signal to NHE1 through ERK kinase. In addition to directly phosphorylating NHE1, ERK is an upstream regulator of p90 Ribosomal S6 kinase (Rsk) which is also identified to phosphorylate NHE1 (S703). Thus in the ERK signaling pathway, there are two protein kinases confirmed to bind, phosphorylate and regulate NHE1 activity. The outstanding question remains; are these kinases acting independently and directly on NHE1 or in the canonical pathway fashion? An initial observation of MAPK (ERK) mediated NHE1 regulation was first made in smooth and skeletal muscle extracts [13]. Support for coordination of ERK and Rsk regulation of NHE1 was identified in acidified cardiacmyocytes [14,15]. In an interesting study, both ERK and Rsk phosphorylation were found to be critical for the activation of invasion and migration in breast cancer cells. Intriguingly, the S703 Rsk site acts independently of the ERK sites where it appears to control movement of tumor cells into and out of the circulatory system [16]. The potential for two kinases from a single signaling pathway to phosphorylate and potentiate NHE1 function makes biochemical sense. However, there are several examples of the independent involvement of these kinases in NHE1

regulation. For example, serum stimulation of NHE1 transport was abolished after a serine to alanine mutation of the Rsk phosphorylation site indicating ERK was needed as an upstream activator of Rsk but not for the direct activation of NHE1 [10]. Inversely, α -adrenergic signaling and acidosis in cardiac cells required ERK but not Rsk for NHE1 activation especially in early signaling events [17] indicating a deviation from canonical signaling pathways in different cell types under different conditions.

The complexity of signaling can also be demonstrated focusing on ERK and other non-growth factor pathway activated kinases. Our research found Rsk and another non-ERK activated kinase, Rock, are both involved in NHE1 activation. Rock was first described as a RhoA mediated activator of NHE1 function in lung fibroblasts [11]. Using dominant-negative constructs and inhibitor studies we found that α -adrenergic and growth factor receptor stimulation required both RhoA/Rock and the ERK pathways for full activation of proton transport [8].

Lysophosphatidic acid (LPA) signals both through RhoA and Rock as well as through a cross-talk mechanism to activate the ERK/Rsk pathway. Because Rsk is downstream of Erk and the site of phosphorylation by Rock was not yet identified, we could not distinguish which of these kinases were responsible for NHE1 activation. In later work, using *in vitro* mass spectrometric analysis of recombinant ctNHE1 we revealed four candidate Rock phosphorylation sites [7]. One site, (T653) was identified as the responsive residue phosphorylated by both Rock I and II isoform. To investigate the role of Rsk and Rock on NHE1 activity we generated full-length human NHE1 with serine to alanine mutations of both sites on NHE1 leaving the ERK site(s) intact in stable transfected fibroblasts. This work showed that both Rsk and Rock kinases independently, but only partially, regulated NHE1 activity.

LPA mediated NHE1 activity was completely blocked when both Rsk and Rock sites were mutated to an alanine indicating a dominance of Rsk vs ERK LPA signaling. Neither Rsk nor Rock phosphorylation alone could fully activate NHE1; however the loss of either phosphorylation site equally abrogated LPA induced cell motility [7]. The importance of ERK/Rsk signaling in NHE1 activity is not universal. In the primary cilium of fibroblasts, growth factor activation (PDGF AA) required the ERK/Rsk pathway for NHE1 mediated migration and cytoskeletal formation but was not involved in NHE1 transport activation [18]. Interestingly in this work AKT, also activated by PDGF AA, was shown to activate both NHE1 transport and cell motility.

Our understanding of the complexity of the regulation of NHE1 by ERK is expanding. Some of the early work discovering ERK phosphorylation identified six different sites (S693, S766, S770, S771, T779 and S785) [19]. Two of these sites S770 and S771 have been shown to be key for ERK activation of NHE1 in sustained acidified AP-1 cells while a third site plays a lesser role (S766) [20]. Mutation of all three sites have been used to show the role of ERK signaling to NHE1 in breast cancer motility and proton exchange [21] while S770 and S771 but not S693 were required for transport activation in cardiomyocytes [17]. These observations were confirmed in structural work where aspartic acid phosphomimetic mutations in S770 and S771 (S770D/S771D) not only activated the exchanger but were required for the conformational changes to a more compact structure that appears to drive increased proton exchange [22].

Recent powerful biophysical and cellular studies show that the intrinsic domain of ctNHE1 is not only the target for ERK but acts as a unique scaffold for the protein kinase [22]. Different than earlier studies, computational and structural biochemical analysis identified six sites phosphorylated by ERK (S693, S723, S726, S771, T779 and S785). Interestingly, previously identified sites, S770 and S766 were not detected to be phosphorylated by ERK in this study but two sites that were identified as ERK sites, S723 and S726, are also ascribed to p38 phosphorylation. The order of binding and apparent binding rate for these sites offer potential insight into the role multiple ERK phosphorylation may play on NHE1. Two of these sites, S693 and T779 were phosphorylated first with the highest K_{app} while S771 was the slowest to phosphorylate NHE1 and did so with a 40 fold lower apparent rate constant than S693 and T779. Each site was bound and modified by ERK independent of the other indicating a non-cooperative role. The biological impact of each site has yet to be determined. This work contradicts the proposed role of ERK in heart cells [3] where both S770 and S771 are required for ERK-mediated NHE1 activation. It is worth pointing out that the lowest K_{app} and last phosphorylated site (S771) was required for NHE1 transport. Complicating our understanding of how this phosphorylation event(s) impact NHE1 is the intrinsic domain of the exchanger. It is possible that some of these phosphorylation sites control structural shifts of the exchanger such that NHE1 wraps itself around the kinase in a dynamic "flexible wrapper" scaffolding influenced by phosphorylation [22].

Other Kinases

The regulatory role of other kinases on NHE1 activity is just as complex and only partially resolved. Interestingly the same site phosphorylated by Rsk (S703) is phosphorylated in a pathway that may involve Sgk1 kinase in cardiomyocytes [23]. In addition to ERK, another MAPK family members, p38 has been reported to phosphorylate and both activate [24] and inhibit [25] NHE1. In prostate cancer cells, induction of the prostate-specific G-protein coupled receptor (PSGR) stimulates NHE1 via two sets of protein kinases, p38 and the FAK non-receptor protein tyrosine kinase (Pyk2). Interestingly in the prostate cells, PSGR lead to p38 phosphorylation by Pyk2 increasing p38 activity. PSGR also increased phosphorylation of NHE1 at two residues [26] by an unidentified kinase. It is possible that Pyk2, one of several kinases activated in this system, could be responsible for this phosphorylation. Similar to the inhibitory effects of p38, AKT is reported to phosphorylate (S648) and inhibit NHE1 activity in acidified hypertrophic cardiomyocytes [27,28].

Contrasting these results, in PDGF stimulated fibroblasts, AKT phosphorylation is required for NHE1 activation and not inhibition [18]. Two additional NHE1 phosphorylation sites S726 and S729 were found to be responsible for part of the apoptotic response to sustained acidosis, however the protein kinases responsible for this have yet to be identified. Nck-interacting kinase (NIK) binds and phosphorylates NHE1 activating transport independent of its conventional downstream effector JNK. Binding and phosphorylation takes place on two different sets of NHE1 residues. NIK binds NHE1 at residues 538-638 and the putative site for NIK phosphorylation lies distal to the binding site [29]. NIK like other kinases phosphorylating NHE1 also target NHE1 regulating proteins such as calcineurin B homologous protein (CHP) and ezrin, radixin, and moesin (ERM) however this relationship is even less understood than NHE1 phosphorylation.

Interestingly, osmotic shrinkage of salamander (*Amphiuma tridactylum*) red blood cells identified changes in phosphorylation at seven sites (S599, S602, T685, S693, S703, S785, S796), only two S703 and S785 have been reported before, however this study hasn't been demonstrated in human cells [30]. Several reports of kinases including PKA, Raf, CamK II and others have been found to bind or phosphorylate NHE1 but the specific residues have not been identified [reviewed in 1-3]. Adding to the complexity is a potential epigenetic regulation of the kinases and phosphatases themselves between tissues or conditions make this challenge even greater. Thus our vision of the intricate phosphorylation landscape of NHE1, while crowded, remains unclear.

Diversity in Signaling

Clearly the relationship between kinases and their signaling to regulate NHE1 is not easily understood by discrete isolated studies using various cell lines or comparisons between one or two kinases. To highlight the diversity in signaling with three agonists; GPCR ligands LPA and phenylephrine (PE) and the growth factor ligand PDGF BB we provide such an initial such investigation using lung fibroblasts stably transfected with full length human NHE1 [7]. To see how LPA signaling involves ERK, Rsk, Rock, AKT and Pyk2 we determined the pHi change in cells treated with kinase inhibitors. We measured pHi after 14 hour serum deprivation using 2',7'-Bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein (BCECF AM) as described earlier but using a plate reader to measure the dual-excitation single emission indicator. Each well was calibrated using nigericin.

Three of the inhibitors diminished the LPA-induced pHi increase due to ERK, AKT, ROCK, and Rsk kinases (Figure 2.). It should be noted that none of these inhibitors completely blocked LPA activation indicating that, as seen in our earlier work, more than one kinase is necessary to fully activate proton transport in lung fibroblasts. Interestingly the FAK Pyk2 inhibitor, PF27632 enhanced agonist stimulation. Starved cells (non agonist controls) displayed a 0.2 pHi unit increase prior to agonist addition (data not shown). It is suggestive that Pyk2 either directly or indirectly is an inhibitor of NHE1 function. One could envision that Pyk2 activates an NHE1 inhibitor (Pyk2 does phosphorylate p38 which has been reported to inhibit NHE1), or Pyk2 regulates a phosphatase that controls NHE1 transport. As such this evidence provides supports the studies performed showing an unknown role of the kinase in prostate cancer cells [26].

To further demonstrate how one kinase is differentially involved in regulating NHE1 activation we treated cells with three agonists in the presence and absence of Y-27632 (Figure 3). As expected Rock activity is required for at least partial NHE1 activation for GPCR agonists PE and LPA. Growth factor stimulation was not significantly inhibited by PDGF. While these data do not show the impact of direct phosphorylation and certainly some of the effect could be to other non-NHE1 mediated events, this work does demonstrate the complex nature of NHE1 regulation and that the coordination and hierarchy of phosphorylation is far from being complete.

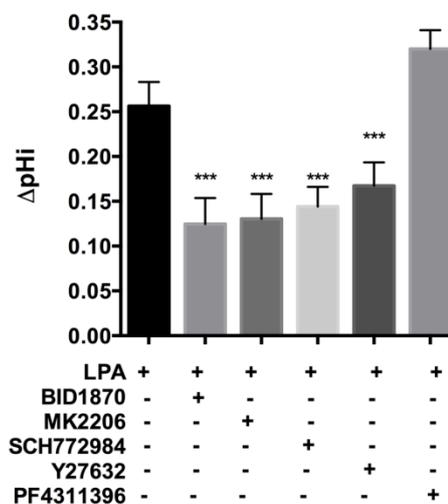


Figure 2: LPA activation of NHE1 Requires more than once protein kinase. Serum starved PSN fibroblasts were incubated with 10 μ M 1-palmitidic-2-hydroxy-3-phosphatidic acid (LPA) for 20 min and the pHi determined in the absence and presence of inhibitor. The following inhibitors were pre-incubated for one hour prior to LPA addition. Inhibitors included 10 μ M Rsk inhibitor (BID1870), 1 μ M AKT inhibitor (MK2206), 0.5 μ M MEK inhibitor (SCH772984), 10 μ M Rock inhibitor (Y27632) and 1 μ M Pyk2 inhibitor (PF4311396). Fold increase in pHi (μ pHi) was determined in control and agonist/inhibitor treated cells. Statistical changes from agonist alone were analyzed using an unpaired, one-tailed T test. ** $P \leq 0.01$ *** $P \leq 0.001$.

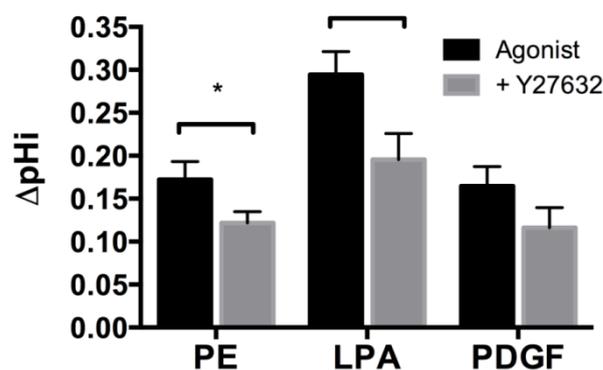


Figure 3: Signal pathway dependence on Rock activation of NHE1 transport. Serum starved cells were treated with either 100 μ M phenylephrine (PE), 10 μ M LPA or 1 ng/ml platelet derived growth factor BB (PDGF) for 20 min in the absence and presence of 10 M Rock inhibitor (Y27632). Fold increase in pHi (μ pHi) was determined in control and agonist/inhibitor treated cells. Statistical change for each agonist alone was analyzed using an unpaired, one-tailed T test. * $P < 0.05$ *** $P \leq 0.001$.

Conclusion

Our understanding of NHE1 regulation has significantly progressed from our initial ctNHE1 deletion studies. Though such work has created more questions than answers, it has helped to advance our understanding of the exchanger. The crucial role NHE1 plays in cell biology homeostasis and disease stresses the importance of having a comprehensive understanding of NHE1 regulation. While we highlight the complex binding, regulation and or ERK-NHE1 interaction this is likely not the only such multifaceted relationship between NHE1 and its kinases. Included in such a comprehensive study it so understand the upstream kinase regulators that impact NHE1 including small G proteins (Rac1, CDC42 and other kinase activators). The obvious need is to identify the remaining phosphorylation sites and where there are conflicting reports or multiple and overlapping sites, to systematically determine the role of each kinase in NHE1 modification. Of particular interest is how individual or a combination of phosphorylation events impact the relationship between the intrinsic disordered domain and the membrane proximal domain to regulate both the scaffolding properties of the ctNHE1 and proton exchange rate or set point for transport of the membrane domain. The differences in Rsk and Erk participation in NHE1 regulation in distinct tissues with a range of agonists also demonstrate the need to be comprehensive in our approach. How each phosphorylation event influences the binding and kinetics of subsequent phosphorylations has only been hinted at by the studies presented here and are far from being fully revealed. An interesting and unknown question is does the phosphorylation of one site alter the ability of a second site to be phosphorylated or possibly could one kinase induce a phosphatase activity at a second site. Adding to the complex nature of NHE1 regulation is the impact of phosphorylation on its binding partners. Several of the protein interacting partners are themselves phosphorylated by the same kinases that modify NHE1. Use of detailed structural work is limited by the nature of a membrane protein. Even with increased access to techniques like cryo-electron microscopy, understanding the complex nature of many posttranslational modifications will require a novel method to decipher completed structures. New biophysical approaches including NMR and mass spec are revealing new sites of interaction and hinting at another level of interactions where interpreting the conflicts between these two approaches will reveal the most interesting questions.

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