Use of BioID to detect protein-protein interactions

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Abstract

Proximity-dependent biotin identification (BioID) is a novel approach to identify protein-protein interactions (PPIs) in a natural cellular environment. BioID exploits a mutant form of a biotin protein ligase found in Escherichia coli, BirA*, that promiscuously catalyzes biotinylation of proteins in close-proximity of the enzyme. Biotinylated proteins are then purified with conventional methods. BioID has been shown to overcome many of the limitations faced by traditional PPI techniques, such as co-immunoprecipitation, proximity ligation assays and yeast two-hybrid systems. The main advantages of BioID as compared to these methods include high sensitivity and spatial resolution, preservation of physiologically- relevant conditions, and detection of weak or transient interactions. Despite some inherent limitations, BioID remains a promising PPI technique and has led to more advanced methods, such as BioID2 and split-BioID.

Introduction

For decades, the genome revolution has contributed to our understanding of human disease, however proteomics has contributed greatly to the field as it allows for large-scale analysis of proteins in order to understand functions of genes implicated in disease. In addition to their complexity, proteins rarely exist in simple complexes and often form interconnected networks in order to exhibit important functions as part of a larger mechanistic pathway. Therefore, understanding their function in the context of protein-protein interactions (PPIs) can provide great insight into the numerous mechanisms underlying disease. Many of the approaches currently used to assess PPIs are done so in an environment different to that in which they naturally occur and often lack the ability to detect weak or transient interactions. Therefore, many proteins remain unrepresented by these conventional methods of screening for PPIs. Here, we discuss an innovative approach for PPI screening called BioID, and highlight the key reasons for its superiority to these conventional methods.

Limitations of common PPI techniques

One of the most widely used techniques for detecting PPIs is co-immunoprecipitation (co-IP), which is often used to test whether two known proteins interact, or to screen for novel protein interactors of a protein of interest (Golemis, 2002). Co-IP harnesses the principle of a specific protein-antibody reaction, in which an antibody against the protein of interest, conjugated to beads, is used to precipitate the protein of interest and its interactor proteins. Subsequent western blotting or mass spectrometry analysis can be performed to blot for a known protein, or to identify novel interactors, respectively. Although this technique allows protein interactions to occur in non-denaturing conditions, if the protein interaction or antibody is weak, certain interactions are often not detected (Golemis, 2002).

Another common PPI screening technique is called a proximity ligation assay (PLA), which uses two primary antibodies raised in different species for two specific proteins of interest (Lin et al., 2015). Secondary antibodies, each conjugated to an oligonucleotide (PLA probe) bind to the primary antibodies and a ligation solution containing two oligonucelotides and a ligase is added. If the proteins are in close proximity, the oligonucleotides in the solution will hybridize to 2 the PLA probes to form a closed circle. Subsequent addition of an amplification solution allows for circular polymerase chain reaction amplification, resulting in a fluorescent signal (Lin et al., 2015). Although this technique allows for visualization of PPIs in

physiologically-relevant conditions, it is not appropriate for large-scale interaction screens (Lin et al., 2015).

The last commonly used technique for PPI screening is the yeast two-hybrid (Y2H) system, in which the interaction between two proteins, the bait and prey, activates a reporter gene that allows for growth of cells harbouring this gene, on specific media (Brückner et al., 2009). A major drawback for this technique includes its inability to detect indirect PPIs, unless a yeast ortholog to the protein exists to mediate the interaction. Additionally, it harnesses an artificial system in which the bait and prey fused to DNA-binding and activation domains are exogenously introduced into cells and therefore does not allow for analysis of endogenous protein interactions under physiological conditions (Brückner et al., 2009).

BirA* as a promiscuous biotin ligase

Upon discovery of a promiscuous biotin ligase, a novel approach for detecting PPIs was established, termed BioID. Originally, BioID was derived from a DNA-protein interaction screening technique, developed by van Steensel and Henikoff, called DamID. DamID uses the fusion of a prokaryotic Dam methylase to a DNA-binding protein, which when expressed in eukaryotic cells will methylate DNA sequences that are in close proximity. This methylated DNA acts as a marker of the interaction that can be subsequently analyzed (van Steensel and Hanikoff, 2000). BioID, similar to the principle of DamID, uses a biotin ligase, called BirA, fused to a protein of interest, that upon expression in mammalian cells will biotinylate any close- proximity proteins. The biotinylated proteins can then be isolated and identified by traditional methods such as mass spectrometry (Roux et al., 2012).

BirA is a 35-kDa DNA-binding, biotin holoenzyme synthetase ligase originally found in Escherichia coli. In the bacterial system, BirA catalyzes the post-translational modifications of transferring biotin to specific lysine residues of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-coA carboxylase (Beckett et al., 1999). This specific class of enzymes, called biotin-dependent carboxylases, undergo post-translational modification in which biotin is covalently linked to a single lysine residue via an amide bond (Beckett et al., 1999). The biotinylation of these lysine-bearing carboxylases occurs in two steps: (1) BirA catalyzes the conjugation of biotin and adenosine triphosphate (ATP) to form a highly reactive intermediate, biotinyl-5'-adenylate (bio-5'-AMP or biotinoyl-5'-AMP). (2) This activated biotin is retained in the BirA active site until it interacts with a specific lysine residue within a target protein, in which an amide bond is formed between the biotin moiety and the lysine residue (Beckett et al., 1999; Roux et al., 2012). This biotinylation reaction, as shown in Figure 1, is highly specific to biotin-dependent carboxylases, however Roux et al. wanted to obtain a more promiscuous biotin ligase as a tool for detecting PPIs. This led to the discovery of a specific BirA mutant (R118G), called BirA*, which is defective in the self-association and DNA-binding, and displays two- orders of magnitude lower affinity for biotinoyl-5'AMP than the wild-type BirA, therefore resulting in premature release of the highly reactive intermediate (Kwon and Beckett, 2000; Streaker and Beckett, 2006). Furthermore, it has been shown that BirA* expression in E. coli results in promiscuous biotinylation of proteins in close proximity - this was further validated in vitro. This led Roux et al. to further study the potential of BirA* to biotinylate proteins in mammalian cells, which eventually led to its use as a tool for PPIs.

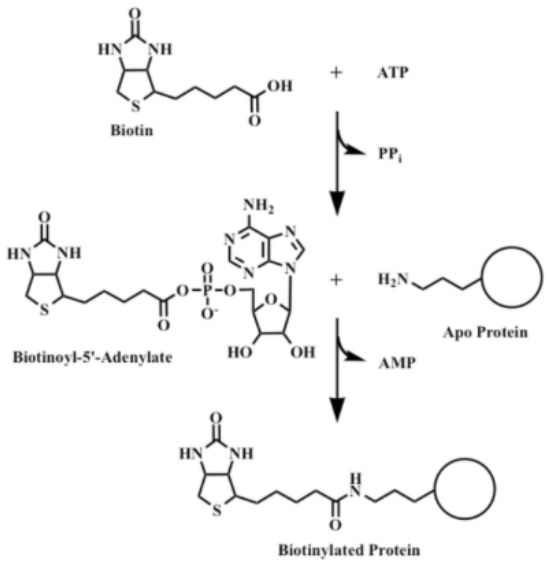

Fig. 1. Biotinylation reaction (Henke and Cronan, 2014). The attachment of biotin to proteins is a two-step process: (1) the biotin ligase, BirA, conjugates biotin and ATP to form a reactive biotinoyl-5'-AMP intermediate, and (2) conserved lysine residues of acceptor proteins, which are nucleophilic, attack the anhydride bond to produce the biotinylated protein.

BirA* function in mammalian systems

To test whether BirA* functions as a promiscuous ligase in a mammalian system, Roux et al (2012) generated myc-epitope tagged BirA-WT (wild type) and BirA*, which they then expressed in HeLa cells. Subsequently, they used streptavidin – which forms an extremely strong non-covalent bond to biotin – conjugated to horseradish peroxidase (HRP), in order to visualize biotinylated proteins via western blot analysis (Roux et al., 2012). This experiment showed that BirA* biotinylated modest levels of proteins as compared with BirA-WT. However, in the presence of 50μm biotin within the HeLa cell tissue culture medium, it was shown that the BirA* promiscuously biotinylated proteins in these mammalian cells (Roux et al., 2012). Therefore, the limiting factor of the level of biotinylation is the concentration of available free biotin, and this biotin must be added in excess to the already present biotin within the fetal calf serum of conventional tissue culture media (Roux et al., 2012). The results of this experiment can be seen in Figure 2.

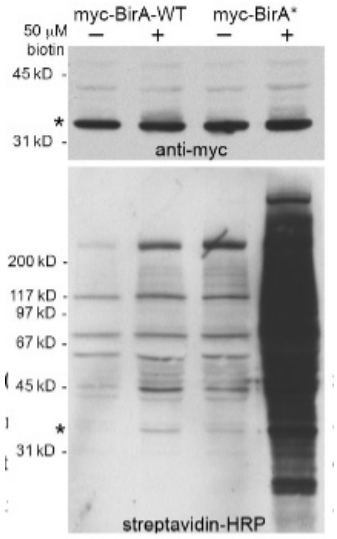

Fig. 2. BirA* enhances biotinylation of proteins in mammalian cells (Roux et al., 2012). HeLa cells were transiently transfected with myc-BirA-WT (wild type) or myc-BirA* (R118G), cultured with or without supplemental biotin (50 μM) and analyzed after 24 hours. Top panel: western blot analysis shows similar levels of the exogenous BirA (asterisk) in samples with anti-myc. Bottom panel: biotinylated exogenous BirA (asterisk) proteins and endogenous proteins were detected with HRP-streptavidin. The myc-BirA* samples showed increased protein biotinylation as compared with the WT isoform (control). This difference is dramatically enhanced by the presence of excess biotin.

In addition to testing the function of BirA* in the mammalian system, Roux et al. (2012) wanted to determine whether BirA* could be used as an experimental tool for identifying close-proximity proteins in vivo. To do this, they generated human kidney fibroblasts (HEK)-293 cells that inducibly express LaA (a constituent of nuclear lamina) N-terminally tagged with myc-BirA*. The cells were subsequently cultured either in the presence (50uM) or absence of exogenous biotin and the biotinylation of endogenous proteins was analyzed via western blot probing with streptavidin-HRP antibody. It was shown, as seen in Figure 3, that the presence of 50uM of exogenous biotin, a large number of nuclear envelope proteins were biotinylated, therefore confirming the biotinylation of close-proximity proteins (Roux et al., 2012).

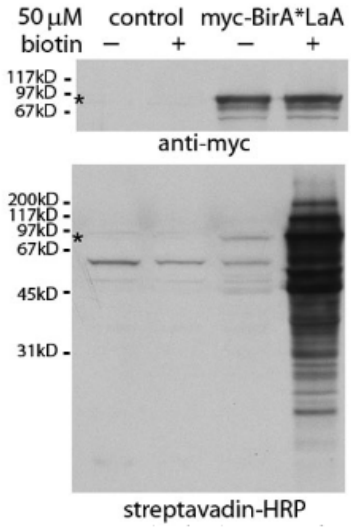

Fig. 3. Proximity-dependent promiscuous biotinylation by BirA*-LaA (Roux et al., 2012). HEK293 cells that inducibly express myc-BirA*LaA or wild-type LaA (controls) were examined after 24 hours of incubation with or without biotin. Western blot analysis of the LaA fusion protein (asterisk) shows detection with anti-myc. The presence of supplemental biotin does not affect the levels of endogenously biotinylated proteins within the control cells. However, this excess biotin significantly increases the biotinylation of endogenous proteins by myc-BirA*LaA.

BioID: novel approach for screening PPI

BirA* is a key player in BioID, a PPI screening technique (Roux et al., 2012). The principle of this system is to fuse a protein of interest to BirA* and subsequently introduce this fusion protein into cells, which are then incubated in excess biotin

(Roux et al., 2013). During this incubation, the BirA* will continuously release the highly reactive molecules of biotinoyl-5'-AMP, leading to biotinylation of any proteins that are in close vicinity of the fusion protein. The biotinylated proteins – direct binding partners and other proteins in close proximity – can then be purified using streptavidin-coated beads (Roux et al., 2013). These beads have a qualitatively high affinity for biotin, forming a non-covalent bond strong enough to withstand extremely harsh lysis conditions. This enables certain proteins (i.e. cytoskeletal proteins) that normally remain insoluble in weaker detergents, to efficiently become soluble and captured (Roux et al. 2013). Therefore, one of the major strengths of BioID is the capacity to maintain important information about PPIs, while utilizing extremely stringent detergents for cell lysis in order to capture proteins such as cytoskeletal proteins (Roux et al., 2013). Upon purification of biotinylated proteins via streptavidin-coated beads, these proteins can be identified by conventional methods such as mass spectrometry, as seen in Figure 4.

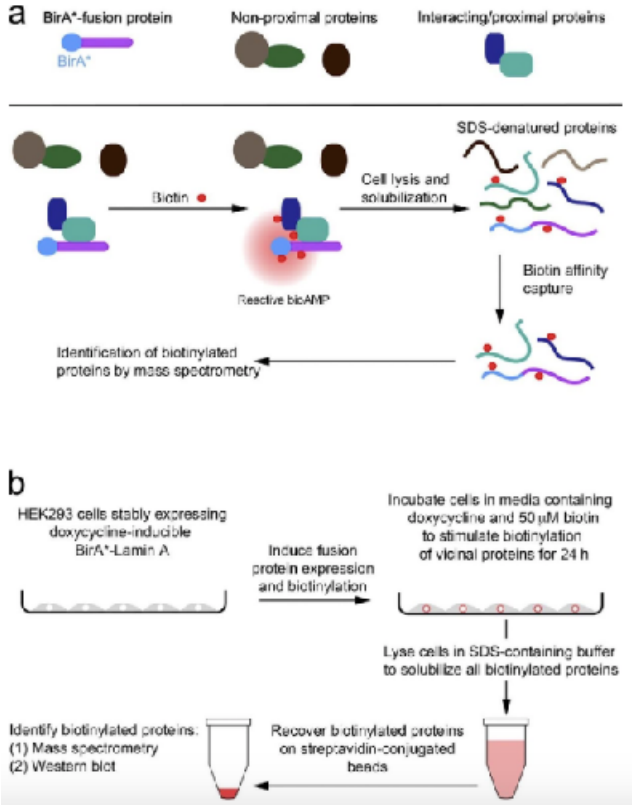

Fig. 4. Model for application of BioID (Roux et al., 2012). (a) Interacting or proximal proteins are tagged and subsequently undergo biotinylation via the promiscuous biotin–ligase, BirA*. Following stringent cell lysis and protein denaturation, affinity purification occurs involving streptavidin beads to pull down the biotinylated proteins. Candidate proteins are often determined by mass spectrometry. (b) Application of BioID to LaA involved the use of HEK293 cells expressing inducible myc-BirA*LaA to test the functionality of BirA* in the mammalian system. Cells were cultured with (50µM) or without exogenous biotin 24 hours before lysis and subsequently lysed under harsh conditions. Streptavidin-conjugated beads allowed for the collection of the biotinylated proteins for later analysis and identification.

Advantages of BioID

Techniques for PPIs, such as PLAs and Y2H systems often face limitations, such that they cannot detect interactions of proteins that contain post-translational modifications or require them for their interactions (Mehus et al., 2015). However, BioID is distinct from these conventional techniques primarily with its ability to detect weak or transient interactions, preserve physiologically-relevant conditions, and identify interactors with high sensitivity (Mehus et al., 2015; Kim, 2016).

Firat-Karalar and Stearns (2015) used BioID to study the protein interactions at the centrosome, translating this work to other researchers who validated the technique's efficacy in screening proximity interactions within mammalian centrosomes. This was due to the strong affinity of biotin for streptavidin, which inevitably allowed for protein purification to occur under stringent denaturing conditions and subsequently preserving the proximal interaction, while solubilizing the centrosome (Firat-Karalar and Stearns, 2015). The direct correlation of conditions promoting protein solubilization and preservation is specific to BioID; often absent in former methods (Roux et al., 2012). Biotinylation precedes solubilization, which allows for weaker and transient interactions to be identified, as outlined by successful detection of BioID-LaA soluble and membrane proteins (Roux et al., 2012).

In non-native environments, which are typically required for PPI techniques such as Y2H systems, proteins and protein fragments are more susceptible to misfolding, relative to their normal cellular context (Roux et al., 2012). However, BioID screens potential vicinal proteins under relatively natural cellular conditions and therefore provides physiologically-relevant information regarding PPIs (Mehus et al., 2015; Kim and Roux, 2016). Although BioID is contingent on exogenous biotin in order for biotinylation to take place

it allows for temporal inducibility of labelling, in experiments where biotinylation process may exhibit a toxic effect.

In addition to its ability to preserve physiological conditions during the point of interaction, BioID allows for detection of biotinylated proteins through the use of streptavidin-coated beads that form strong bonds with the biotin of the modified proteins (Kim, 2016). As such, biotinylation enables the BioID system to reach a maximal level of isolated protein purification. Consequently, this results in greater sensitivity and spatial resolution, as well as low background resolution (Kim, 2016). Additionally, BioID enables detection of low abundance proteins, being a technique that can successfully identify novel PPIs and insoluble constituents that often evade traditional methods (Roux et al., 2012).

Other proximity-labeling techniques, such as selective proteomic proximity labeling assay using tyramide (SPPLAT) and proximity labeling with ascorbate peroxidase (APEX) utilize tyramide-based reagents that covalently attach to aromatic amino acid side chains, such as tyrosine, and hydrogen peroxidase to initiate labeling of neighbouring proteins (Rees, 2015). Sufficient labeling of the protein target is often prevented by formation of constituent aggregation and further impeded by the reagents' properties, as they cannot easily detect individually modified proteins (Rees, 2015). BioID's ability to label lysine residues, which are not only more abundant than tyrosines but also more structurally exposed, circumvents this problem. Furthermore, the labelling frequency when using these tyramide-based reagents is likely to be lower due to its dependency on covalently coupling to aromatic groups, and thus the exposure of these residues. However, tyramide-based proximity labelling techniques perhaps provide even further advantages due to the shorter half life of the tyramide-based reagents as compared to biotin-adenylate ester that are essential for BioID labelling, and thus requires shorter incubation periods (Li et al., 2017). Despite these advantages, BioID is deemed for its simplicity and lack of toxic labeling conditions, as it does not require hydrogen peroxidase to initiate labelling, universal to tyramide-based methods (Branon et al., 2017).

Caveats of BioID

As with any novel technique, comes a number of inherent drawbacks. In any proximity-dependent labeling system, one must consider the accessibility for protein interactions to occur, as the number of proteins, structure and orientation play a vital role in successfully screening these neighbouring proteins (Kim and Roux, 2016). In addition to the inability for BioID to assess the strength of PPIs, the amount of biotinylated proteins is also not a valid means to identify true protein association, as low amounts may be more biologically relevant than those of higher quantities (Mehus et al., 2015; Kim and Roux, 2016; Roux et al., 2012). Furthermore, the protein of interest can also be structurally changed by fusion to BirA*. Independent of the protein's size, the fusion of a tag can affect the protein's structure and stability, alternating its interactions and ultimately its function (Mehus et al., 2015). Additionally, during the irreversible covalent modification of biotinylation of primary amines, site-specific charge loss and alterations of the labeled proteins can arise, which can impair localization and function of the fusion protein as well as vicinal proteins (Kim and Roux, 2016; Roux et al., 2012). This can therefore result in an inaccurate representation of potential interactors. However, this limitation can be overcome by first avoiding alteration of the protein of interest's function by targeting the C or N-terminal regions and subsequently testing the functionality of the final BirA*-fused protein of interest, if the function is known (Roux, 2013).

In addition to the technical limitations of BioID, validation is required to determine whether the protein of interest is directly interacting with the biotinylated proteins or if the detected proteins are merely in the vicinity of the protein of interest. Due to this, there are also several caveats within the interpretation of BioID results. For example, positive interactors that result from the BioID screen do not prove that there is a direct interaction, as labeled candidates may reside in close proximity of the BirA*-fused to the protein of interest, but not physically interact with it (Roux, 2013). Furthermore, false negatives can also arise as a result of true interactors that lack the proximate primary amines required for the biotinylation process (Kim and Roux, 2016).

Finally, biotin is transported into mammalian cell cytoplasms and depends on diffusion into the nucleus (Zempleni, 2005). Despite biotin being non-toxic, the addition of excess biotin as seen in normal conditions, can influence protein function when

incubated for an extended labelling time (Kim and Roux, 2016).

Applications of BioID in other biochemistry techniques

Although the original purpose for developing BioID was to utilize it as a tool for identifying PPIs, its capacity for detecting close-proximity proteins allows for elucidation of meaningful information about protein dynamics and can be combined with other biochemistry tools such as crosslinking and immunoprecipitation followed by high-throughput sequencing (CLIP-seq) in order to unveil different proteomic networks.

In addition, the BirA*-fused proteins of interest can be integrated into the genome of the cell line of interest through homologous recombination via CRISPR-Cas9 genome editing (Ran et al., 2013). By designing homologous arms on either end of the BirA*-protein of interest fusion repair template, the tagged protein of interest can be inserted into the genome of the cells in order to endogenously express these BirA* tagged proteins. The combination of BioID with CRISPR-Cas9 systems provides a mechanism for detecting PPI in physiologically-relevant conditions and thus overcomes this challenge that limits many other PPI techniques.

Future Directions of BioID

BioID2

Kim et al. (2016) recently discovered a more efficient method, termed BioID2. It elaborates on the former system by employing a significantly smaller promiscuous biotin ligase, and in turn efficiently labels close-proximity proteins. Sun2, a type II nuclear envelope (NE) protein, is susceptible to endoplasmic reticulum (ER) mislocalization, like other NE proteins. It does so particularly when its N-terminus is fused to structurally large elements. As depicted in Figure 5a, functional validation of both enzymes showed that BioID2 facilitated appropriate expression of Sun2 at the NE, relative to the ER, at a magnitude of two-fold, in comparison to BioID. This confirms that the reduction in tag size allows for improved functionality. Aside from this, BioID2 possesses a lower optimal temperature and remarkably requires over 15 times less biotin than BioID for promiscuous biotinylation. The introduction of flexible linkers increased the labeling radius of BioID2 to thereby improve identification of proteins, initially refractory to the former BioID system (Kim et al., 2016).

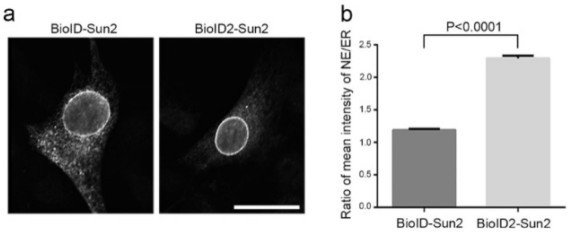

Fig. 5. BioID2 enhances fusion protein localization (Kim et al., 2016). (a) Mouse fibroblast NIH3T3 cells were used to transient express NE protein, Sun2 with BioID or BioID2, marked as BioID-human Sun2 and BioID2-human Sun2, respectively. More appropriate localization was facilitated by BioID2-human Sun2. Scale bar: 10μM. (b) The NE/ER ratio is based on the men intensity detected with anti-human Sun2. 48 nuclei/condition were measured and values represent the mean + SEM.

Split BioID

In addition to improving the efficacy of BioID, a modification on the original system has also been developed as a way to detect PPIs between two known proteins of interest. More specifically, De Munter and colleagues (2017) experimented with fusing half of BirA* to one protein and the other with a different protein. They found that when these proteins are in close proximity to one another, the BirA* halves fuse to form a functional BirA*, in which subsequent biotinylation reveal both proteins as potential interactors. The only caveat of this modified BioID system is its similarity to complementation assays, such as bimolecular fluorescence complementation, which introduces tagged proteins exogenously, and therefore prevents analysis of protein dynamics in physiologically-relevant conditions. However, use of CRISPR-Cas9 system can resolve this issue by integrating the BirA*-protein of interest fusions into the genome for endogenous expression.

Conclusion

BioID has laid the foundation for novel proximity-dependent identification techniques, paving the way for further improvements and future discoveries. The ability to endogenously tag a protein of interest with BirA*, or the smaller biotin ligase employed by BioID2, via CRISPR-Cas9 systems, provides a natural

model for studying PPIs. Additionally, the ability for BioID to maintain physiologically-relevant conditions and detect weak or transient interactions, while still utilizing harsh lysis conditions to capture proteins that would otherwise be insoluble, presents a powerful advantage over other conventional PPI techniques.

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