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Recent Developments in Halotag Technology

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Abstract

Several biological functions and eukaryotic proteomes are interrelated and they depend upon protein function and analysis. Protein function and interaction is a biological process. Deviations in these interactions can occur. In order to overcome this problem, scientists have devised a fusion platform called HaloTag technology. It is a technique used for functional analysis of proteins. This technique involves development of a 33kDa HaloTag that is fused to protein of interest and development of HaloTag ligand. When the HaloTag fusion protein tag and ligand come in contact, a covalent bond is formed rapidly. The covalent bond formed is irreversible so it ensures efficient isolation and purification of target protein. Variety of ligands is available for analysis of protein. Proteins always react differently to the new therapeutic products and it is unpredictable to discover their role after interacting with the ligand. HaloTag technology is used for analysis of protein-protein interactions, protein DNA interactions, protein functional analysis, enzyme immobilization and tumor cell detection. HaloTag technology evaluated the function in a way that it captures only protein of interest that is captured and purified.

Introduction

HaloTag technology is comprises of two main elements, the HaloTag protein, a protein fusion tag that can be fused to any protein of interest (POI) genetically and number of various organic molecules, named as HaloTag ligand that binds irreversibly to the HaloTag protein.

High impurity as well as low yield has confined the traditional systems for tagging of protein. The conformational and functional alterations can be done by high molecular weight. The Polyhistidine tag has small size. It does not alter protein functions so it is highly recommended for protein analysis. It serves as important tool for protein isolation but has high impurity because of non-specific binding of other proteins. Histag is effective only for protein isolation and purification so it has been observed that other methodologies should be discovered and applied for cellular imaging [1].

The complexity between protein-protein interactions has limited the research in finding new diagnostic as well as curative or treatment options for various diseases. Moreover, ineffective methods for labelling of protein have restricted the proteomic analysis. Protein purification is an important process but it is time consuming, therefore, new methods are being discovered so that analysis of protein can be done efficiently [2] in Figure 1.

This technology involves two steps including the development of a 33kDa HaloTag that fuse to protein of interest and secondly the development of a HaloTag ligand [3]. When HaloTag protein and ligand come in contact with each other, covalent bond is formed rapidly and is irreversible. Its mechanism relies on the bacterial haloalkane dehalogenase enzyme from Rhodococcus rhodochrous, in which the substitution of Phe272 is done by His272 [4].

The HaloTag technology has overcome several drawbacks of traditional protein tagging systems because it performs complete protein analysis by utilizing single genetic construct [5].

The procedure for cell based applications involves

- Sample imaging as live or fixed cells
- Expression of fusion chimera in cells
- Labelling of cells with particular HaloTag ligand
- Formation of a vector that encodes the fusion of HaloTag protein to a protein or protein-domain [6].

When enzyme and particular ligand come in contact, formation of an alkyl-enzyme intermediate takes place when nucleophilic displacement of terminal chloride occurs with Asp106. It has been reported that as substituted Phe272 do not catalyze the hydrolysis so this reaction changes in mutant dehalogenase where it results in a covalent adduct of high stability in Figures 2 and 3 [7].

The HaloTag technology serves many benefits as compared to the traditional methods for protein tagging. Single genetic construct is required as many HaloTag ligands are available to study various characteristics of the target protein. Secondly, bond between HaloTag protein and ligand is irreversible thus it allows sequential labelling experiments to examine the mechanism of protein synthesis as well as degradation. As the HaloTag complex has the ability to remain relatively stable under drastic conditions so it can be helpful for imaging of live as well as fixed cells [8].

The efficacy of the HaloTag technology helps in making it more favourable for various protein analysis applications in vitro and in vivo. This review explains all applications of HaloTag platform for isolation and purification of protein, protein function analysis, studying protein-protein as well as protein-DNA interactions, and in vitro and in vivo cellular imaging [9]. Different HaloTag ligands are available these days for several applications. As the covalent bond formed between HaloTag ligand and protein is irreversible so it ensures rapid isolation as well as purification [10]. Some of the examples of HaloTag ligands includes the HaloTag resins for the immobilization of the proteins, Alexa Fluor 488 for the labelling of cell surfaces and TMR ligand for the labelling cytosolic proteins [11].

Overview

There are two elements belong to HaloTag technology which includes:

HaloTag protein (A protein which forms a fusion tag and goes to fuse genetically with any protein which is of interest (POI).

HaloTag Ligands (There are different varieties of organic molecules which bind in irreversible manner to HaloTag proteins).

The ligands should interact with HaloTag proteins specifically and efficiently.

Therefore, the ligands are divided to two elements which include:

Constant group (reactive)

Variable group (functional or reporter) [12]

The constant or reactive group contains a chloroalkane. This is important for the attachment of substrate to the HaloTag protein covalently. As, this region is constant and conserved therefore these groups have the constant properties for all the ligands [13].

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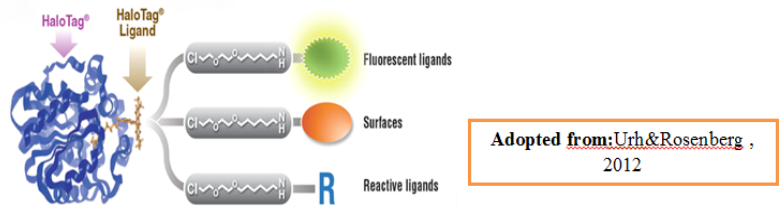


Figure 1. Representation of a HaloTag system (constant and functional groups are present in ligands).

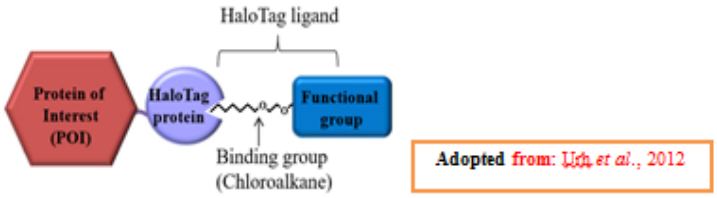


Fig.4: General HaloTag system

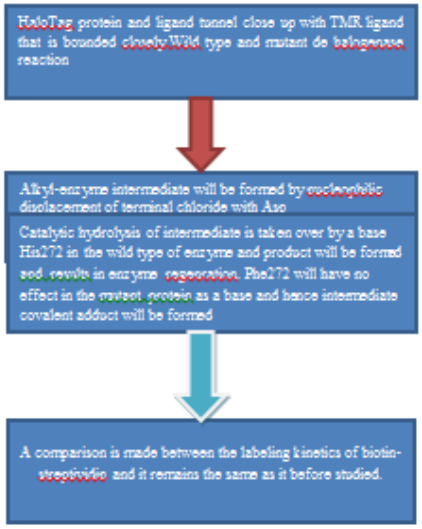


Figure 2. General Halotag System.

HaloTag System

Generally, HaloTag includes the HaloTag protein and differential functional groups are present alongwith selected HaloTag ligands. Protein of interest (POI) gets fused to HaloTag protein easily. The HaloTag ligands bind to the HaloTag fusions with covalent bonds and are specific in nature. In this example there is studied the expression of HaloTag protein and ligand of wild and mutant type dehalogenase on protein expression. The intermediates were formed and their catalysis was studies by adding bases which effects the reaction in wild but there was no effect on mutant type intermediate. A comparison studies shows that the reactions remain the same as were before [14] Figures 4 and 5.

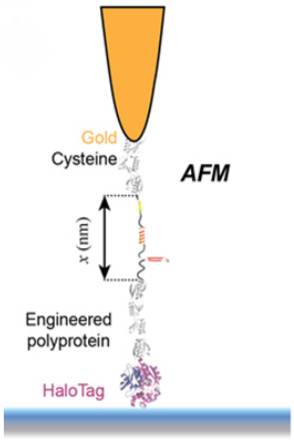
Nanomechanics of HaloTag tethers

Halotag which is an artificially developed HaloalkaneDehalogenase can form ester bonds with a chloro-alkane functionalized surface was used with thiol chemistry to achieve this goal. For the covalent attachment of proteins to surface, the procedure of using a Halotag is becoming popular.

As an example we can look at the successful use of fusion proteins and terminal Halotag to label live cells and purify proteins with increased purity and high yield. Another example is the use of Halotag in single molecule force spectroscopy to immobilize poly Filamin to beads through DNA spacers and poly 127 polyprotein to mica surfaces [15] Figures 6 and 7.

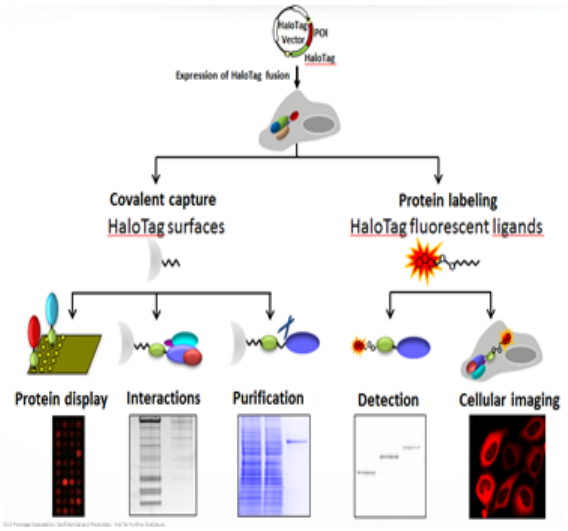
Although covalent anchoring extends the lifetime, has high detachment forces, increased tethering time and increased range of forces to which a single molecule can be exposed, due to the little knowledge about Halotag its potential use in single molecule experiments is limited. The denaturation of Halotag under certain conditions poses both advantages and disadvantages. One advantage of Halotag as compared to other covalent attachment techniques is its mechanical fingerprint caused due to its extension up to its chloroalkane anchoring point and unfolding. The number of trapped amino acids within the protein fold and the direction of the pulling force must also be taken into consideration when looking at the response of Halotag to the denaturing forces. The interference of Halotag on the studied process might pose the biggest disadvantage for this process [16]. Investigation into the effects of the refolding and unfolding of two model proteins and the methods employed to attach proteins using thiol chemistry and HaloTag-chloroalkane anchoring is reported [17]. Analytical tools were developed to verify the specific HaloTag attachment to chloroalkane-functionalized surfaces. Unfolding mechanisms of Halotag caused by force-clamp AFM and force extension are discussed here. By uncoupling its refolding and unfolding from these proteins of interest, the mechanical properties of Halotag are used to show the the usage of HaloTag to investigate mechanical properties of other proteins. Through measure folding of Halotag fused polyproteins and Halotag anchoring a guide for successfully employing covalent attachment of polyproteins is provided protein-protein interaction.

Protein-Protein interaction occurs during the cellular signalling while protein-DNA interaction requires complex techniques. The HaloTag technology has limited the research in order to save time allowing scientists to visualize the interaction among intracellular and extracellular transmembrane proteins by using micro patterning techniques. Halotag technique adaptation helped



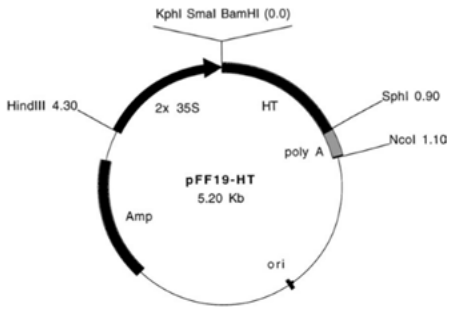
Adopted from: Oreo *et al.*, 2017

Figure 3. HaloTag protein in AFM (Atomic force microscopy).



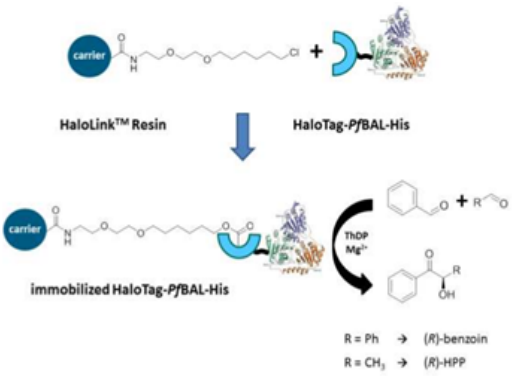
Adopted from: Kovacevic, 2013

Figure 4. Relevant Protein interactions by covalent-capture HaloTag technology.



Adopted from: Lang *et al.*, 2006

Figure 5. pFF19-HT vector for HaloTag transformation.



Adopted from: Pohl, 2016

Figure 6. Immobilization of enzyme through covalent one step evaluation.

them to examine the epidermal growth factor receptor present in living cells to measure the dissociation contents of several protein complexes. Halotag has been used for the investigation of interactions between protein-DNA by using highly effective methods for the analysis of human genes.

Halo-Tag in plants

In tobacco and some popular cells the expression of Halotag technology was observed which was then followed by staining using the diAcFAM ligands and the HaloTag TMR. Efficient fluorescence was generated in different cell types of plants due to HaloTag technology proving that it is a flexible system. From CaMV-35S, poly A tail sequence and double enhancer along with CaMV-35S control which carries HaloTag protein cDNA in the high copy pBluescript-based plasmid for studying the transformation of tobacco protoplast and it helps to localize the HaloTag proteins which are non-fluorescent in Figures 8 and 9.

Immoblization of enzymes

The Hallotag™ system from Promega, unlike most of the fusions that mediate non-covalent attachment to respective supports, enables covalent bonding without using aggressive cross-linking agents e.g. glutaraldehyde. Halo-alkane dehalogenase from *Rhodococcus rhodocrous*has a specifically designed suicide variant HaloTag. It forms an ester bond to an aspartate residue in the active site enabling a site-directed, gentle and fast immobilization taken directly from the extracts of crude cell and it identifies terminal chloro-alkanes displayed on any carriers quickly. HaloTag has its main application in various biomedical applications e.g. cell imaging and the in vivo detection of protein interactions. After criticism faced on an early publication containing the study of this tag using a monomeric lipase, HaloTag was optimized and it lead to the formation of a new variant. Other reports potray mostly the positive effects of this variant on the soluble production of recombinant enzymes. The goal here is to use the latest HaloTag version. It acts as a generic strategy for the immobilization of enzymes. Here are some of the examples of the first results on the thiamine diphosphate-dependent homotetramericbenzaldehydelyase produced from Pseudomonas fluorescence (PfBAL), an important catalyst for the production of 2-hydroxy ketones, to demonstrate the functionality of the HaloTag strategy also for complex enzymes Figures 10 and 11.

Expression and purification of CARM1

There are at least 10 representatives of proteins within mammals which are protein arginine methyl transferases (PRMT). The transfer of methyl group is catalyzed by these proteins to diverse protein targets from adenosylmethionine (SAM). Due to this methylation of surface diverse cellular processes are affected which includes transcriptional regulation, RNA splicing and DNA damage repair. Glycine-arginine repeats (GAR) which are part of

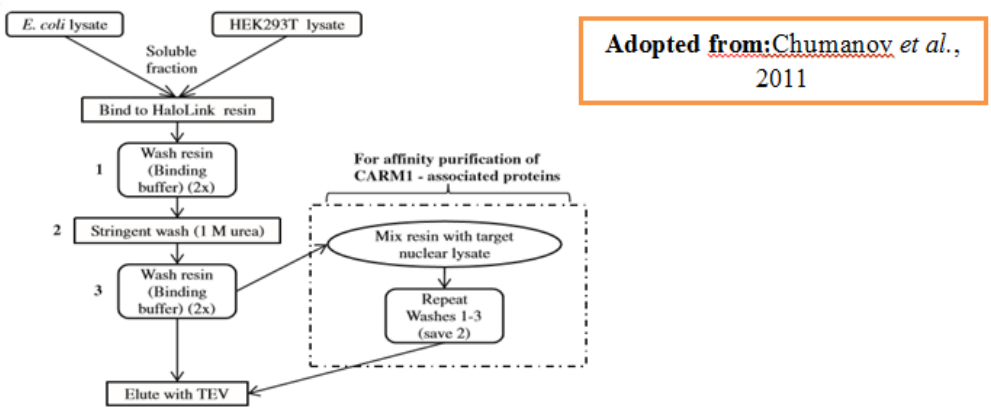


Figure 7. CARM1 tagged N-terminal HaloTag expression and purification from *E.coli*.

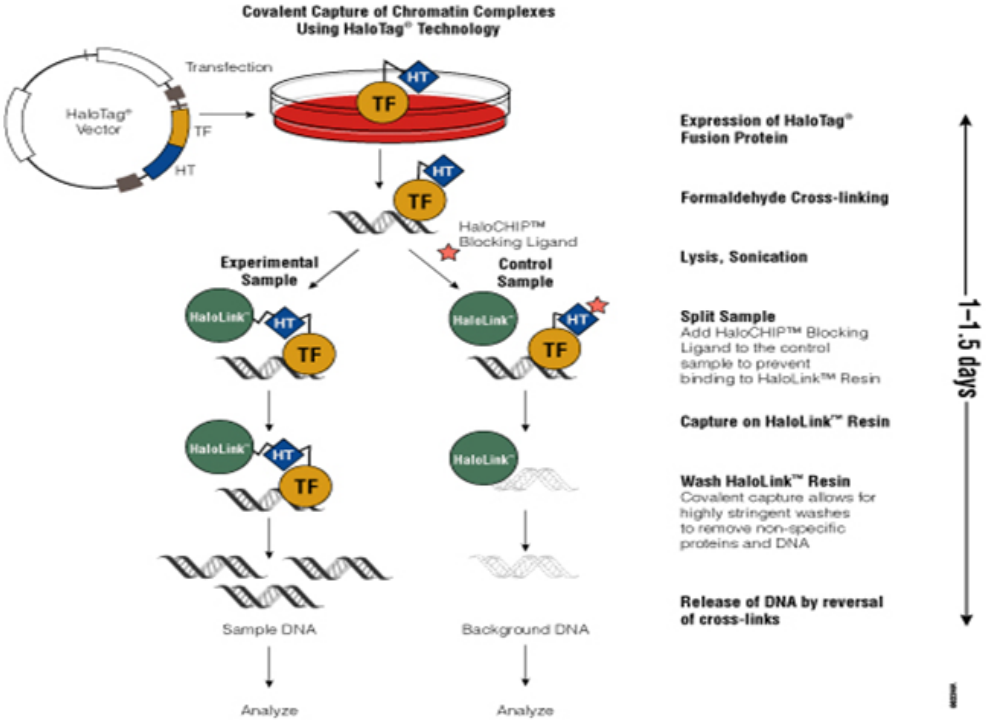


Figure 8. Use of antibodies for chromatin immunoprecipitation.

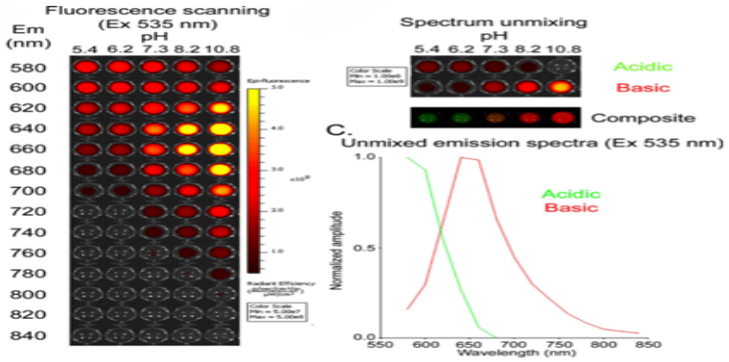


Figure 9. At different pH, SNARF-ligandounmixing gives different fluorescence patterns.

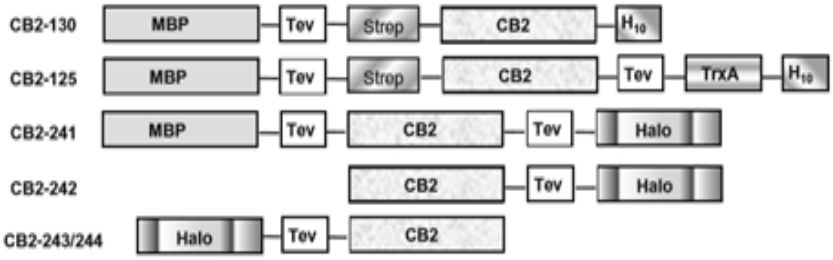


Figure 10. Controls of CB2 and CB2 fusion proteins, plasmids 243 and 242 are different from one another in resistance of antibiotics and promoters type.

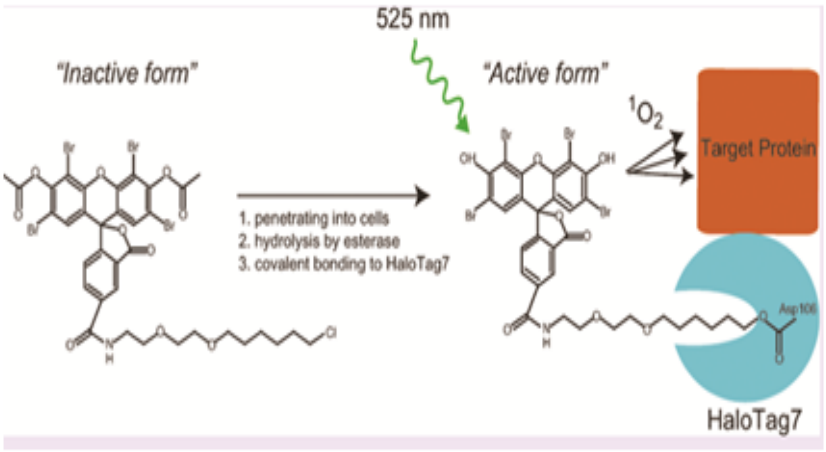


Figure 11. The conversion of inactive form of the compound in active form.

proteins in substrate are mostly methylated by PRMT family. In the arginine side chain, one or more groups of methyl are transferred to terminal amino group from SAM by the PRMT enzyme in methylation reaction.

CARM-1(coactivator-associated arginine methyl transferase 1) also known as PRMT4 is considered as transcription activation co-activator. Homodimers are formed by CARM1 utilized for catalytic activity and CARM 1 forms the largest PRMT enzyme and contains 608 amino acids. CARM1 is novel and unique because GAR motifs are not methylated by it. GAR methylates a wide range of sequences which are not similar. The core for catalysis of CARM1 is highly conserved and 150-470 amino acids are present in it. 28-508 spanned amino acids structures has been solved. For transcriptional activity, 480-608 amino acids are important but not included in catalytic activity. HaloTag technology also interprets the expression of proteins solubilized in *E.coli*. CARM1 protein has been purified in full length within mammalian cells.

Protein-DNA interactions

Designing of well-defined structures is favored by the incomparable specificity of various sequence of DNA oligonucleotides and base pairing. Oligonucleotides are practical for use in multiple applications due to their mechanical stability and ready availability. Effective drug delivery is enabled by the therapeutic application of conjugates composed of proteins or peptides and single stranded DNA. (Wang *et al*., 2012). DNA-protein and protein-protein interactions are also an application of this. The fabrication of protein arrays also use DNA-protein conjugates which provide universal and reversal binding surface, enhanced binding capacity and increased reversibility.

Bio analytical immune assays also use DNA-protein conjugates to help in signal generation. The ultrasensitive immune assays have been enabled by immuno-RCA (rolling circle amplification) and immuno-PCR (polymerase chain reaction) by measuring the amplification product formed by conjugated oligonucleotide. Analyte recognition uses two separate oligonucleotide-binder conjugates which are used in proximity ligation assays. Template is formed by the ligation of two sequences for nucleic acid amplification after the analyte-bound antibodies bring the conjugated oligonucleotide sequences. Using direct luminescence-based detection reporter-labeled oligonucleotide-binder conjugates have been used. The production of the DNA-protein conjugates and their high performance in assays is enabled by conjugation chemistry. A widely used and well established method is the covalent conjugation using cross-linkers to primary amine or sulfhydral groups of the protein. Because of the availability of several sites in a protein the conjugation is uncontrollable. The amount of oligonucleotide per antibody may vary from batch to batch and the random oligonucleotide conjugation to a protein may interfere with analyte recognition. The modification of recombinant binders to enable site-

specific coupling and stoichiometric conjugation overcomes this problem. DNA-protein conjugations to allow stoichiometric and site-specific are covalent in nature and for these reasons ligands have been developed. Biomolecules which are connected show high affinity of streptavidin and biotin. The nature of streptavidin is tetrameric due to which conjugation stoichiometric ally gets challenged. The conjugation of protein-oligonucleotide has been employed and developed with unaffected binding affinity of monovalent streptavidin. Via selective enzymatic self-labeling enables covalent coupling by modified enzymes such as CLIP-tag, HaloTag and SNAP-tag. The reaction of the synthetic ligand with the modified enzymes results in a covalent bond. The use of protein tags in versatile applications such as studies in protein-DNA, protein-protein, protein emission tomography, protein purification and tumor detection. The specific conjugation of proteins to oligonucleotides also uses the tags. Self-labeling can be performed in living cells which makes the protein tags to be useful in studies of cellular functions.

Protein-labeling for tumor cells detection

There came a hallmark of HaloTag technology for the labeling of proteins in-vivo (fluorescently). There was development in the haloalkane dehalogenase in a bacteria that is modified .The synthetic ligands can be linked covalently to the protein tag. The labeling of protein tag and ligand is irreversible and interact specifically with the tag protein.

The ligand consists of linker which is a chloroalkane and a group which is functional in nature. They can easily permeate through membranes and are smaller in size.

Modules for pH sensing SNARF-1™ has been used in HaloTag ligands. The pH changes are monitored through this module intracellularly. This technology travelled from in vitro to in vivo applications and first of all a tag was fluorescently labeled which was expressing cancer cells and environment of acidic pH was exposed on to it during tumorogenesis. Xenograft model of human colon cancer by the expression of HaloTag HCT116,Standard fluorescent ligand (NIR) and SNARF-1™ ligands for pH sensing provens the principle that after systematic delivery of fluorescent ligands there occurs tag specific labeling.

Protein function analysis

Proteins always react differently to the new therapeutic products and it is unpredictable to discover their role after interacting with the ligand. Halotag technology evaluated the function in a way that it captures only protein of interest is captured and purified. This experiment was performed on a stable cell line that was expressing HaloTag-PCSL9. This technique included two types of HaloTag ligands including permeable and impermeable ligands. Using this technique revealed that this specific protein possesses a single membrane spanning domain. This technique proved to be superior then fluorescent labeled proteins.

Cannabinoid receptors CB2 expression and function

CB1 and CB2 are cannabinoid receptors and they belong to long family of g protein coupled receptors. CB2 is expressed by cells of our immune system and is considered the primary target to cure several disorders including immune disorders and inflammatory diseases. Moreover, its primary function appears to be cAMP accumulation inhibition. HaloTag technology is used to improve CB2's yield in equilibrium based chromatography step. This technique involves the use of inactive HaloTag from *Rhodococcus sp.* This HaloTag binds covalently to the chloroalkane ligand. The bond formed is covalent and irreversible. As the binding is irreversible, extensive washing steps can be applied that will help to improve its purity and yield. Thus the HaloTagged protein can be immobilized in the resin and thus can be easily purified and eluted from the resin.

Protein degradation

The enormous applications and analyses are invaluable for knowing directed at the elucidation of protein purpose. Such studies are frequently complemented by approaches that effort to attain specific elimination of the action of proteins in an effort to recognize the underlying mechanism leading a particular biological organization. Traditionally, methods such as mutagenesis, genetic knock-out technology, RNA interference (RNAi) andantisense RNA have been used to specifically eliminate a function of a particular protein. However, these methods are all indirect effectors of the protein of interest and suffer from genetic compensation, low time resolution and can be difficult to utilize in live animals. HaloTag brings a new analytical device for precise and direct protein reduction or inactivation of protein function at the protein level Studies showed that binding of HaloTag ligands containing a hydrophobic moiety to HaloTag fusion proteins destines these proteins for efficient proteosomal ruin. This novel hydrophobic tagging technology of HaloTag fusions efficiently induced degradation of proteins in cell culture systems, as well as directly in animals, potentially making this system ideal for validating potential drug targets in disease models.

Fusion proteins inactivation

CALI is defined as chromophore assisted light inactivation. It allows the functional analysis of the protein of interest by disrupting the protein within the living cells with high resolution. This technique involves the use of chromophore molecule as photosensitizer which yields short lived reactive oxygen species when irradiated with intense light. CALI has not been used significantly because of its limitations. These limitations include low production of reactive oxygen species and difficulty in labelling of protein of interest with photosensitizer. Currently, flourescein is being used as photosensitizer because it shows high production of ROS. Eosin is used as photosensitizer that shows 11 fold more production of ROS than flourescein. Thus, for covalent labelling of eosin with protein of interest, a specific membrane permeable eosin ligand is synthesized for HaloTag technology that shows easy labelling and inactivation of HaloTag fused protein in the living cells. This technique is valid for many cell biological assays within the living cells.

Conclusion

Proteins always react differently to the new therapeutic products and it is unpredictable to discover their role after interacting with the ligand. HaloTag technology is used for analysis of protein-protein interactions, protein DNA interactions, protein functional analysis, enzyme immobilization and tumor cell detection. HaloTag technology evaluated the function in a way that it captures only protein of interest that is captured and purified.

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