Live-Cell Targeting of His-Tagged Proteins by Multivalent N-Nitrilotriacetic Acid Carrier Complexes

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Supporting Information

ABSTRACT: Selective and fast labeling of proteins in living cells is a major challenge. Live-cell labeling techniques require high specificity, high labeling density, and cell permeability of the tagging molecule to target the protein of interest. Here we report on the site-specific, rapid, and efficient labeling of endogenous and recombinant histidine-tagged proteins in distinct subcellular compartments using cell-penetrating multivalent chelator carrier complexes. In vivo labeling was followed in real time in living cells, demonstrating a high specificity and high degree of colocalization in the crowded cellular environment.

Live-cell labeling and tracking of proteins with therapeutic relevance pose a significant challenge. – 3 To understand the interplay of proteins and cellular pathways in cells, a range of fluorescent reporters have been explored. Live-cell labeling demands high specificity, high labeling density, and cell permeability of the tagging molecule to track the protein of interest (POI) in the crowded cellular environment at a protein concentration of 300 ng/mL. Large efforts have been made to site-specifically incorporate chemical reporters such as small organic dyes, genetically encoded amino acids, or autofluorescent proteins to label, track, and manipulate specific targets. Selective tools such as probe incorporation mediated by enzymes (PRIME) and the fusion proteins SNAP, CLIP, and Halo emphasize these efforts. Organic probes are preferred over autofluorescent proteins because of their significantly smaller size, superior quantum yield, photostability, and broader spectral range. Small tags, such as the tetracysteine tag, the retro-Diels–Alder reaction of tetrazines with alkenes, N-nitrilotriacetic acid (NTA), or its multivalent derivative trisNTA have further contributed to the analysis of structure, function, and dynamics of proteins. The (sub)nanomolar affinity of the trisNTA moiety toward His-tagged proteins enables the site-specific and stoichiometric labeling of POIs. In contrast to monoNTA, trisNTA offers kinetically stable binding while the small size of the trisNTA/His-tag interaction pair keeps the influence on the physiological function of the POI as low as possible. Both properties are of special interest for super-resolution microscopy, single-molecule tracking, and other emerging techniques. The trisNTA/His-tag interaction pair has unique potential for highly specific labeling of POIs in living cells and intracellular delivery of functional His-tagged proteins in vivo. However, its poor cell permeability has limited its in vivo applications to date.

Several technologies have been established for the intracellular delivery of proteins, nucleic acids, therapeutic agents, or other chemical reporters to study and perturb biological networks. Cell-penetrating peptides (CPPs) have often been used to target cytosolic proteins without harming the cell. One of the first identified CPPs was the transactivator of transcription (TAT) of HIV-1, which is responsible for cellular uptake and translocation into the nucleus. The basic domain of TAT (pRKKRRQRRR-) has been identified to be responsible for cell penetration. Since then, the TAT sequence has been conjugated to other proteins to facilitate fast and efficient internalization. In addition to TAT, other CPPs have been identified, including polyarginines, Penetratin, Transportan, and cyclic arginine-rich peptides. The low cytotoxicity of CPPs and the high diversity of the delivered cargoes make them an attractive choice for intracellular delivery. Here, we describe a cell-penetrating trisNTA/His6-TAT49–57 carrier complex to internalize fluorescent trisNTA moieties for the efficient and rapid intracellular targeting of His-tagged proteins in mammalian cells.

We non-covalently linked HIV TAT49–57 to Ni(II)-loaded trisNTA by forming a stoichiometrically defined carrier complex. HIV TAT49–57 (RKKRRQRRR-) equipped with six N-terminal histidines separated by a short flexible GGGS linker (Scheme 1) was synthesized using solid-phase Fmoc chemistry [see the Supporting Information (SI)]. For sensitive detection, the trisNTA was modified with different fluorophores, e.g., ATTO656 or AlexaFluor 647 (AF647). After Ni(II) loading, the trisNTA/His6-TAT49–57 carrier complex was formed to deliver the fluorescent multivalent chelator into the cytosol and nucleus. Once there, the trisNTA preferentially bound to the His6-POI and released the TAT49–57 carrier peptide because of its higher affinity. As previously shown, the affinity of trisNTA toward a His10-tagged protein is 10-fold higher (Kd = 0.1 nM) compared with a His6 tag, but the complex stoichiometry remains 1:1. This allows for efficient and rapid exchange of the trisNTA carrier complex in vivo.

Here we focused on two medically relevant proteins located in distinct cellular compartments. First, we selected the antigen
translocation complex TAP, which resides in the endoplasmic reticulum (ER) membrane. Within the adaptive immune response, TAP shuttles proteasomal degradation products into the ER for loading of major histocompatibility complex (MHC) class I molecules.\textsuperscript{33} Second, the DNA-silencing complex methyl CpG binding protein 2 (MeCP2) localized in the nucleus was investigated. MeCP2 is known to bind to methylated DNA, and mutations in MeCP2 are linked to neurodevelopmental disorders (Rett syndrome).\textsuperscript{34} For colocalization studies, both POIs were fused to an autofluorescent protein, TAP\textsuperscript{mVenus} and MeCP2\textsuperscript{GFP}.\textsuperscript{35,36} Notably, MeCP2 harbors an endogenous sequence of seven consecutive histidines in an unstructured C-terminal region, whereas the TAP construct displays a recombinant C-terminal His\textsubscript{10} tag.

First, human cervical cancer (HeLa) cells expressing membrane-bound TAP\textsuperscript{mVenus}−His\textsubscript{10} were fixed, permeabilized, and incubated with only 100 nM trisNTA\textsubscript{57} carrier complex to target the His\textsubscript{10}-tagged POI in the ER membrane. Confocal laser scanning microscopy revealed a perfect colocalization (Pearson coefficient 0.90) of the His\textsubscript{10}-tagged TAP and trisNTA\textsubscript{57} carrier complex (Figure S1 in the SI). Notably, cells that did not express TAP\textsuperscript{mVenus}−His\textsubscript{10} did not show any fluorescence signal in the trisNTA\textsubscript{57} channel, indicating the specificity of the intracellular labeling. Similar results were obtained for the MeCP2 construct (Figure S2). Here, C2C12 mouse myoblasts expressing MeCP2\textsuperscript{GFP} in the nucleus were labeled with trisNTA\textsubscript{AF647} as noted above. Colocalization of the fluorescence signals emanating from both reporter dyes (GFP and AF647) was detected (Pearson 0.78), demonstrating the applicability of the trisNTA staining and full access of this internal naturally occurring His tag for subsequent live-cell labeling studies.

After demonstrating the site-specific labeling of His-tagged POIs in fixed cells, we carried out cellular uptake and colocalization studies in living cells. We anticipated that the trisNTA carrier complex would be taken up by cells and delivered trisNTA to the POI for labeling, as has been seen for other non-covalent TAT adducts. To address this, we performed in vivo labeling experiments of TAP\textsuperscript{mVenus}−His\textsubscript{10} and MeCP2\textsuperscript{GFP} by tracing the uptake of the trisNTA/His\textsubscript{6}−TAT\textsubscript{49−57} carrier complex in living cells. HeLa cells expressing TAP\textsuperscript{mVenus}−His\textsubscript{10} were incubated with 500 nM trisNTA\textsubscript{ATTO565}/His\textsubscript{6}−TAT\textsubscript{49−57} carrier complex. After transduction for 1 h, site-specific labeling was imaged using confocal laser scanning microscopy. To facilitate the uptake of trisNTA/His\textsubscript{6}−TAT\textsubscript{49−57} in the nanomolar range, cells were preincubated with 0.25 mM chloroquine (1 h at 37 °C) prior to the transduction step. Chloroquine is an inhibitor of endosomal sequestration and is known to increase the transduction efficiency.\textsuperscript{37} Representative images of the live-cell labeling of His\textsubscript{10}-tagged TAP by complex formation with trisNTA\textsubscript{ATTO565} demonstrate specific ER staining (Figure 1; see Figure S3 for further examples).

Colocalization of the fluorescent signals for the His\textsubscript{10}-tagged TAP1 and the red-emitting trisNTA\textsubscript{ATTO565} provided evidence for the site-specific and high-affinity labeling in living cells by the minimal lock-and-key element.

Similar results were obtained for the in vivo labeling of the transcription regulator MeCP2\textsuperscript{GFP} (Figure 1). Transiently transfected C2C12 mouse myoblasts were incubated with the trisNTA/His\textsubscript{6}−TAT\textsubscript{49−57} carrier complex (9 μM) and the uptake was imaged 30 min after addition. In this case, the trisNTA was conjugated with an AF647 dye for readout. Merging the red channel of trisNTA\textsubscript{AF647} with the green channel of MeCP2\textsuperscript{GFP} revealed colocalization of the small lock-and-key element and thus the site-specific targeting of the endogenous His-tag at heterochromatic regions of chromosomes (chromocenters). In all our live-cell approaches, strong ATTO565 or AF647 fluorescence of the trisNTA was observed that perfectly coincided with the fluorescence of the POI in the green
channel (see the SI for further images). No colocalization of the trisNTA/His6-TAT49−57 carrier complex was detected in cells expressing a TAP construct without the His tag or a truncated MeCP2 lacking the intrinsic heptahistidine sequence (Figure S4). It is important to note that no uptake of fluorescently labeled trisNTA was observed in the absence of the TAT49−57 carrier peptide (Figure S5) or in complex with a cumulative histidine sequence lacking the TAT49−57 peptide (His peptide; Figure S6). In addition, the formation of the carrier complex is essential for cellular uptake, since no transduction of the trisNTAs was observed upon removal of the coordinated Ni(II) by addition of EDTA (Figure S7).

Finally, we followed the cellular uptake and labeling of POIs in real time by live-cell confocal microscopy. Imaging of MeCP2GFP in transiently transfected C2C12 cells and subsequent treatment with the preformed trisNTA/His6-TAT49−57 carrier complex demonstrated rapid uptake within 10−30 min (Figure 2). Labeling of the cumulative histidines of MeCP2 proved to be fast and colocalization of the two fluorophores was already detected in the nucleus after 10 min. The two dyes accurately overlapped, indicating that our labeling approach enables efficient, rapid, and site-specific targeting of POIs in living cells. Notably, Ni(II) ions complexed to trisNTA at the given concentrations and incubation periods were nontoxic for the cells, as observed in long-term cell cultures and real-time tracking analysis of His-tagged targets within the interior of living cells.

ASSOCIATED CONTENT

Figure 2. Site-specific targeting of MeCP2GFP by cell-penetrating trisNTA carrier complexes followed in real time. MeCP2GFP (green)-transfected C2C12 cells were treated with the trisNTA54−64/His6-TAT49−57 carrier complex and analyzed by live-cell microscopy. Colocalization of trisNTA54−64 (magenta) and MeCP2GFP was already detected after 10−30 min, demonstrating rapid and site-specific targeting of the POI by trisNTAs (merged channel). Scale bar: 5 μm.

REFERENCES

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