Cell Surface HaloTag® Technology: Spatial Separation and Bidirectional Trafficking of Proteins

ABSTRACT The ability to specifically label proteins within living cells can provide important information about protein dynamics and function. Here we use a technology to covalently tether fluorophores with different wavelengths to the specially designed HaloTag® reporter protein. We have achieved surface expression of the HaloTag® reporter protein by fusing it to a truncated integrin. In addition, we have developed a novel membrane-impermeant fluorophore. By using differently colored cell-impermeant and permeant fluorophores, we show spatial separation of membrane and intracellular protein pools, respectively. The truncated integrin protein, labeled with distinguishable fluorophores, can be followed in real time to study translocation of surface and intracellular protein pools. Using a HaloTag®-integrin fusion protein, we have shown the HaloTag® technology to be a powerful tool to study spatial separation and real-time translocation of protein pools in live cells.

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Promega currently offers a variety of HaloTag® ligands, including the HaloTag® Coumarin, Oregon Green®, diAcFAM, Alexa Fluor® 488, TMR, PEG-Biotin and Biotin Ligands.

INTRODUCTION

Specific labeling of proteins in live cells can provide details about protein function and dynamics within complex intracellular environments. The development of methods for labeling proteins by genetic fusion is expanding the understanding of cellular components. However, visualization of multiple pools of the same protein through space and time is technically challenging with reporter proteins such as green fluorescent protein. The HaloTag® technology[(a–c)] permits tethering of fluorophores with different wavelengths to a reporter protein using a single genetic construct (1). The HaloTag® reporter protein is an engineered catalytically inactive derivative of a bacterial hydrolase that forms a covalent bond with HaloTag® ligands. The HaloTag® ligands contain two crucial components: 1) a common HaloTag® reactive linker, which forms a covalent bond with the HaloTag® protein, and 2) a functional reporter such as a fluorescent dye or affinity handle such as biotin. Here we broaden the use of the HaloTag® technology to show that the HaloTag® reporter protein can be expressed on the cell surface and that fluorescent HaloTag® ligands can be made cell-impermeant. These new developments enable the separation of membrane-associated and intracellular protein pools and the real-time analysis of protein translocation.

SURFACE-DISPLAYED HALOTAG® PROTEIN

We have previously shown that the HaloTag® protein can be fused to a protein of interest and the fusion protein can be properly directed to specific subcellular locations. For example, specific localization to the nucleus was achieved when three copies of a nuclear localization sequence $(NLS)_3$ from the simian virus large T-antigen were added to the HaloTag® protein (2,3). In this article, we describe a cell surface-displayed HaloTag® protein and a cell surface-specific ligand to visualize spatial separation and real-time translocation of distinct protein pools.

We achieved surface expression by fusing the HaloTag® reporter protein to the extracellular domain of truncated β1 integrin (β1Int) downstream of the signal sequence (Figure 1, Panel A; 4). This β1Int-HaloTag® fusion protein was successfully expressed and was well tolerated by multiple cell types, including CHO, HeLa, HEK293, U2OS and human neural stem cells (5). Immunocytochemistry using antibodies against HaloTag® protein and β1 integrin to label nonpermeabilized cells showed that the β1Int-HaloTag® fusion protein was expressed on the cell surface in a pattern similar to that of endogenous β1 integrin (Figure 1, Panel B).

We used the HaloTag® PEG-Biotin Ligand[(a,b)] to confirm that the HaloTag® protein on the cell surface was functional. HEK293 cells stably expressing the β1Int-HaloTag® fusion protein were labeled with HaloTag® PEG-Biotin Ligand, followed by streptavidin conjugated to Alexa Fluor® 488. The same cells were then labeled with the cell-permeant HaloTag® TMR Ligand[(a,b)]. Live-cell imaging showed the HaloTag® PEG-Biotin Ligand and streptavidin-Alexa Fluor® 488 distinctly labeled the cell surface, and the cell-permeant HaloTag® TMR Ligand labeled intracellular β1Int-HaloTag® protein (Figure 1, Panel C). Together, these results showed that the HaloTag® protein is exposed on the cell surface and, importantly, this surface-displayed HaloTag® protein is functional since it can successfully bind to HaloTag® ligands.

CELL SURFACE-SPECIFIC LIGAND

We developed a cell-impermeant fluorescent HaloTag® ligand to specifically label surface-displayed HaloTag® protein in live cells (Figure 2, Panel A). The new HaloTag® Alexa Fluor® 488 Ligand[a–c] showed no detectable cellular toxicity or morphological side effects under the recommended labeling conditions described in the *HaloTag® Technology: Focus on Imaging Technical Manual* #TM260, as assessed by the CellTiter-Glo® Luminescent Cell Viability Assay (data not shown). Live-cell imaging showed that the HaloTag® Alexa Fluor® 488 Ligand specifically labeled the cell-surface HaloTag® protein in HEK293 cells stably expressing the β1Int-HaloTag® protein (Figure 2, Panel B) but did not label intracellular HaloTag® protein in HEK293 cells stably expressing HaloTag®-$(NLS)_3$ protein (Figure 2, Panel C). In addition, multiplexing experiments that combine live-cell labeling and immunocytochemistry are possible because HaloTag® ligands retain their fluorescent properties after fixation. Live HEK293 cells stably expressing the β1Int-HaloTag® protein were labeled with the HaloTag® Alexa Fluor® 488 Ligand, then fixed but not permeabilized. Since cells were not permeabilized, the Anti-HaloTag® polyclonal antibody (pAb; Cat.# G9281) labeled only cell-surface protein. An overlay of green fluorescence from live-cell labeling and red fluorescence from immunocytochemistry showed the novel HaloTag® Alexa Fluor® 488 Ligand localized to the cell surface (Figure 2, Panel D). A portion of the β1Int-HaloTag® protein labeled with the HaloTag® Alexa Fluor® 488 Ligand was also inside the cell, which represents surface protein that was rapidly internalized.

SPATIAL SEPARATION IN LIVE CELLS

Live-cell labeling with the cell-impermeant HaloTag® Alexa Fluor® 488 Ligand, which specifically labels cell-surface proteins, followed by labeling with the cell-permeant HaloTag® TMR Ligand, which labels intracellular proteins, can show the β1Int-HaloTag® protein in two distinct protein pools (Figure 3, Panel A). β1Int-HaloTag® protein-expressing cells pulsed with the HaloTag® Alexa Fluor® 488 Ligand and chased with the HaloTag® TMR Ligand can show two distinct protein pools when analyzed by SDS-PAGE (Figure 3, Panel B, lane 4). Using glycanase treatment, we have shown that the higher molecular weight, green cell-surface pool of β1Int-HaloTag® protein is heavily glycosylated compared to the red intracellular pool (manuscript in preparation). The HaloTag® Alexa Fluor® 488 Ligand alone labeled only the glycosylated protein pool since this ligand specifically labels HaloTag® protein at the cell surface (lane 3). The HaloTag® TMR Ligand alone labeled both protein pools since this ligand labels the cell surface and inside (lane 2). The SDS-PAGE migration pattern for the two pools of β1Int-HaloTag® protein is consistent with the expected glycosylation for integrins,

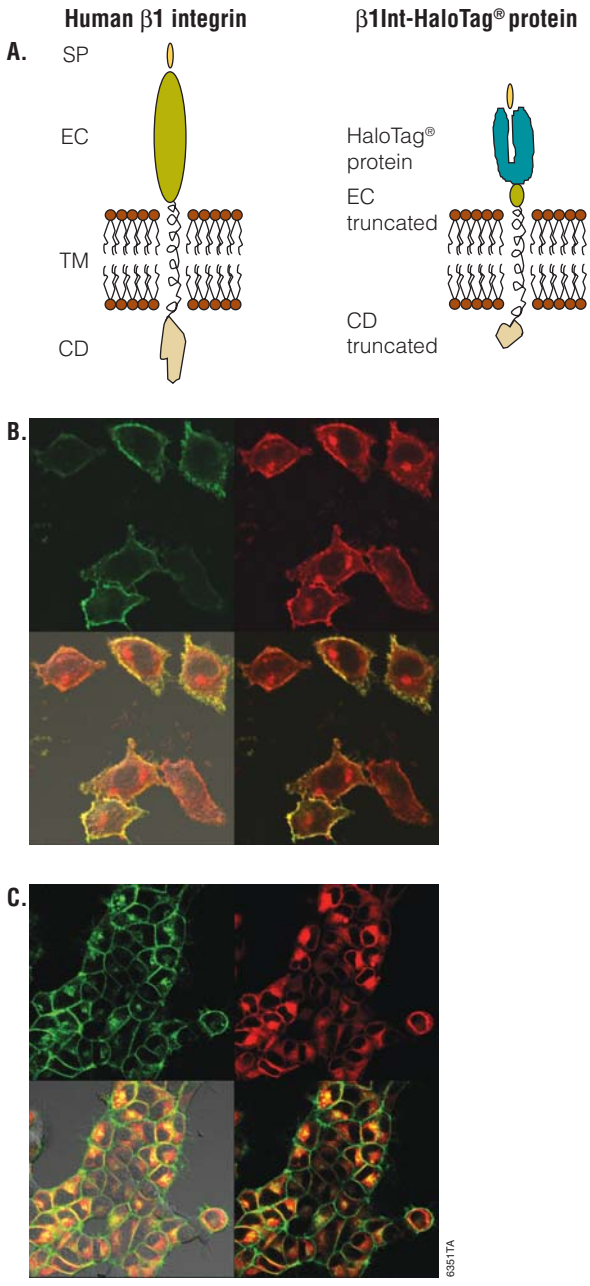

Figure 1. Targeting HaloTag® protein to the cell surface. Panel A. (left) Human β1 integrin is depicted with a signal peptide (SP), extracellular domain (EC), transmembrane domain (TM) and cytoplasmic domain (CD). **Panel A.** (right) In the β1Int-HaloTag® construct, the HaloTag® protein is displayed on the cell surface by fusion with the truncated extracellular domain of human β1 integrin. **Panel B.** HeLa cells transiently expressing the β1Int-HaloTag® protein were fixed and processed for immunocytochemistry without permeabilization. The Anti-HaloTag® pAb was used as described elsewhere (6) and detected using an Alexa Fluor® 488-conjugated anti-rabbit antibody (1:1,000, Molecular Probes). A β1 integrin antibody was used and detected using an Alexa Fluor® 594-conjugated anti-mouse antibody (1:1,000, Molecular Probes). **Panel C.** HEK293 cells stably expressing the β1Int-HaloTag® protein were labeled with HaloTag® PEG-Biotin Ligand (10µM for 15 minutes at 37°C), rinsed, labeled with Alexa Fluor® 488-conjugated streptavidin (1µg/ml for10 minutes at 37°C), rinsed, and labeled with HaloTag® TMR Ligand (5µM for 15 minutes at 37°C). Cell images were generated on an Olympus FV500 confocal microscope in sequential mode using the appropriate filter sets.

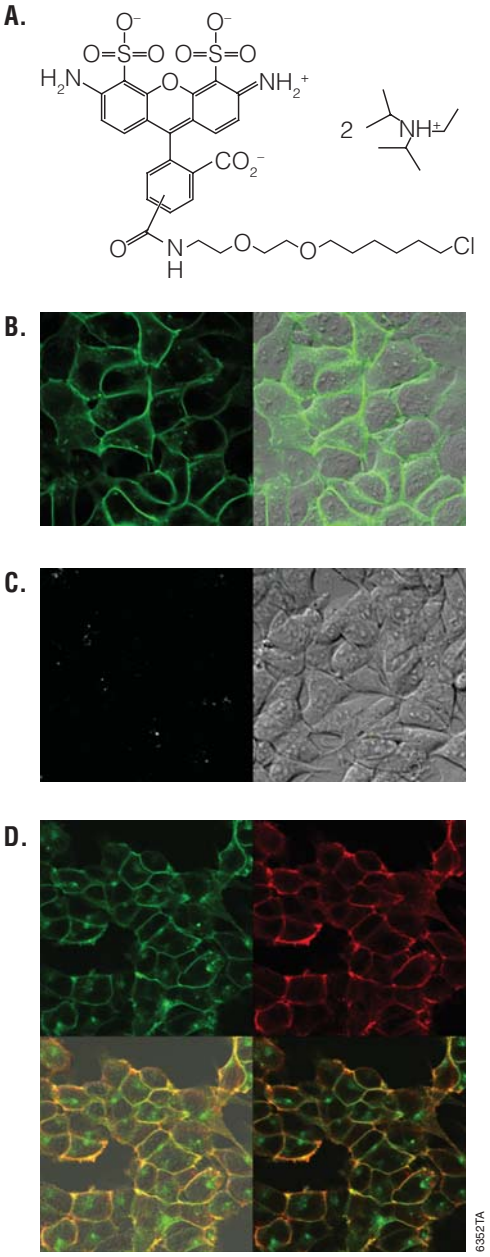

Figure 2. Novel HaloTag® Alexa Fluor® 488 Ligand is cell-impermeable. Panel A. Structure of the HaloTag® Alexa Fluor® 488 Ligand. **Panels B and C.** Live-cell imaging of HEK293 cells stably expressing the β1Int-HaloTag® protein (Panel B) or HaloTag®-$(NLS)_3$ protein (Panel C) labeled with the HaloTag® Alexa Fluor® 488 Ligand (1µM for 15 minutes at 37°C). **Panel D.** HEK293 cells stably expressing the β1Int-HaloTag® protein were labeled with the HaloTag® Alexa Fluor® 488 Ligand, then fixed for immunocytochemistry without permeabilization. The Anti-HaloTag® pAb was used as described elsewhere (6) and detected using an Alexa Fluor® 594-conjugated anti-rabbit antibody (1:1,000 dilution, Molecular Probes). Cell images were generated on an Olympus FV500 confocal microscope using the appropriate filter sets, and fixed cell images (Panel D) were taken in sequential mode.

suggesting that the β1 integrin fused to HaloTag® protein is being processed in a physiologically appropriate manner.

Imaging of live cells pulsed with the HaloTag® Alexa Fluor® 488 Ligand and chased with the HaloTag® TMR Ligand also showed the β1Int-HaloTag® protein in two distinct protein pools. HeLa cells transiently expressing the β1Int-HaloTag® protein showed spatial separation of the cell-surface protein labeled specifically with the HaloTag® Alexa Fluor® 488 Ligand and intracellular protein labeled with the HaloTag® TMR Ligand (Figure 3, Panel C).

REAL-TIME TRANSLOCATION IN LIVE CELLS

The HaloTag® technology can distinguish between two protein pools spatially and separate two protein pools temporally. Live-cell labeling with a HaloTag® Alexa Fluor® 488 Ligand pulse and a HaloTag® TMR Ligand chase showed real-time translocation of differentially labeled proteins as the red cytoplasmic protein pool moved to the cell membrane and the green cell-surface pool internalized (Figure 3, Panel D). In addition, live-cell labeling followed by immunohistochemisty showed that the membrane protein was translocating via the expected intracellular trafficking machinery (manuscript in preparation). This and SDS-PAGE results suggest that fusing the HaloTag® reporter protein to a membrane protein does not significantly alter post-translational modification or protein transport in the cell.

CONCLUSION

The ability to label and analyze membrane proteins in their native environment is critical to develop a detailed understanding of protein trafficking to and from the membrane. The HaloTag® technology is a flexible system that enables efficient labeling of fusion proteins in living cells. By fusing the HaloTag® protein to the extracellular domain of a truncated β1 integrin, we demonstrated that the HaloTag® reporter protein can be expressed on the cell surface and used to study trafficking of a protein in living cells. By developing a novel cell-impermeant dye to specifically label cell-surface proteins, membrane protein pools can be clearly distinguished from intracellular protein pools in living cells. Differently colored cell-impermeant and cell-permeant HaloTag® ligands permits spatial separation of membrane and intracellular protein pools by both SDS-PAGE analysis and live-cell imaging experiments. In addition, real-time translocation of surface proteins can be followed in live cells. In conclusion, the HaloTag® technology provides the ability to separate protein pools in space and time and can be used as a powerful tool to examine the cellular biology of integrins and other membrane proteins of interest.

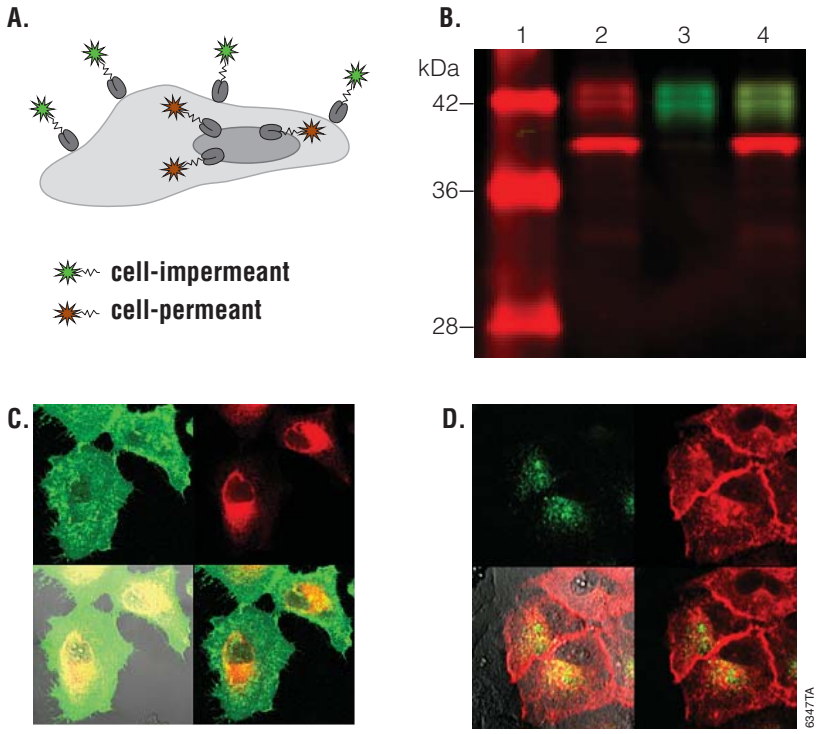

Figure 3. Spatial and temporal separation of proteins using HaloTag® technology. Panel A. Schematic diagram of a cell expressing the β1Int-HaloTag® protein that was pulsed with cell-impermeant HaloTag® Alexa Fluor® 488 Ligand and chased with cell-permeant HaloTag® TMR Ligand to label surface and internal HaloTag® protein, respectively. **Panel B.** HEK293 cells stably expressing the β1Int-HaloTag® protein were labeled with HaloTag® TMR Ligand alone (lane 2), HaloTag® Alexa Fluor® 488 Ligand alone (lane 3) or pulsed with HaloTag® Alexa Fluor® 488 Ligand to label membrane protein and chased with HaloTag® TMR Ligand to label intracellular protein (lane 4). Following live-cell labeling, cells were lysed and analyzed by SDS-PAGE with a molecular weight marker (lane 1). **Panel C.** HeLa cells transiently expressing the β1Int-HaloTag® protein were pulsed with HaloTag® Alexa Fluor® 488 Ligand (1µM for 15 minutes at 37°C), chased with HaloTag® TMR Ligand (5µM for 15 minutes at 37°C), rinsed and imaged with an Olympus FV500 confocal microscope in sequential mode using the appropriate filter sets. **Panel D.** Twelve hours after cell labeling, imaging showed the differentially labeled proteins as they moved from the cytoplasm to the membrane and as they internalized from the membrane.

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ORDERING INFORMATION

Product	Size	Cat.#
HaloTag® Alexa Fluor® 488 Ligand	30µl	G1001
HaloTag® TMR Ligand	30µl	G8251
HaloTag® Coumarin Ligand	30µl	G8581
HaloTag® Oregon Green® Ligand	30µl	G2801
HaloTag® diAcFAM Ligand	30µl	G8272
HaloTag® PEG-Biotin Ligand	30µl	G8591
Anti-HaloTag® pAb	200µg	G9281

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