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Mechanism-based small molecule probes for labeling CD38 on live cells

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Mammalian CD38 is a type II transmembrane protein that converts nicotinamide adenine dinucleotide (NAD) to cyclic adenosine diphosphate-ribose (cADPR, Figure 1A) and ADP ribose.[1] cADPR is a second messenger that can trigger Ca^{2+} release from internal Ca^{2+} stores.[2] Interestingly, CD38 also signals as a receptor.[3] In response to its ligand CD31[4] or agonistic CD38 antibodies, it not only mediates cell adhesion events,[5] but also initiates transmembrane signaling,[6, 7] resulting in the phosphorylation of intracellular proteins such as ERK and Cbl[8, 9] and the upregulation of cytokine production.[10, 11] There is sustained interest in understanding the chemistry and biology of CD38 because it is involved in multiple biological processes, including social behavior,[12] insulin secretion,[13, 14] and immune cell differentiation.[15] Clinically, CD38 expression is a negative prognostic marker for chronic lymphocytic leukemia.[16, 17]

To study CD38 biology, methods to monitor CD38 dynamics in ligand-induced signaling processes are needed. Immunofluorescence and green fluorescent protein (GFP)-fusion are the commonly used methods.[18-21] These methods may affect the binding of or signaling induced by natural ligands. It is known that some CD38 antibodies interfere with its binding to CD31.[4] We therefore sought to develop a labeling method for CD38 using small molecules. Due to their smaller size, small molecule tags are less likely to interfere with ligand-induced signaling.

Our labeling method is based on Schramm and coworkers' discovery that arabinosyl-2′-fluoro-2′-deoxy nicotinamide mononucleotide (F-araNMN) can covalently label CD38 on residue E226 (Figure 1C).[22, 23] We reasoned that if a fluorescently tagged arabinosyl 2′-fluoro-2′-deoxy NAD (F-araNAD) can still form a covalent bond with E226 of CD38, CD38 should be fluorescently labeled (Figure 1C).

We designed and synthesized two F-araNAD compounds conjugated with Rhodamine (Rh), Rh-6-(F-araNAD) (Figure 1D) and Rh-8-(F-araNAD) (Supporting Information, SI). These two compounds were prepared using click chemistry[24-26] from F-araNAD compounds bearing

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Supporting Information Available: Experimental methods and Figure S1 to S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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propargyl groups and Rh bearing an azido group (Figure 1D and supporting information). To test whether Rh-6-(F-araNAD) and Rh-8-(F-araNAD) can covalently label CD38, we first used purified CD38 extracellular domain. Both wild type (wt) and the E226D catalytic mutant (negative control) were incubated with Rh-6-(F-araNAD) or Rh-8-(F-araNAD). The reaction mixtures were resolved by SDS-PAGE. Before staining with Coomassie blue, the gel was visualized under ultra-violet (UV) light to detect Rh fluorescence. Both Rh-6-(F-araNAD) and Rh-8-(F-araNAD) can label wt, but not the E226D mutant of CD38 (Figure 2A), demonstrating that Rh-6-(F-araNAD) and Rh-8-(F-araNAD) are mechanism/activity-based probes for CD38. The labeling reaction is efficient, completing within minutes, and is more efficient than F-araNMN (Fig S1 and S2, Supporting Information). Since both compounds labeled CD38 equally well, for later studies, we only used Rh-6-(F-araNAD). The covalent label has a half life of >40 hour (Fig S3).

We next tested whether Rh-6-(F-araNAD) can label CD38 on the membrane of live cells. We used the human leukemia cell line HL-60. HL-60 cells normally do not express CD38, but CD38 expression can be induced by retinoic acid (RA).[27] Thus HL-60 cells were used as the negative control, while RA-treated HL-60 cells were used as the positive control in the labeling. A stably transfected HL-60 cell line that constitutively expresses a high level of CD38 (CD38H) was used as another positive control.[28] Cells were incubated with 5 μM Rh-6-(F-araNAD) for 30 min at 4°C and then examined by confocal fluorescence microscopy. As shown in Figure 2 (B-D), HL-60 cells without RA-treatment showed no fluorescent labeling, while RA-treated cells and CD38H cells were both labeled. In addition, Raji cells naturally expressing CD38 can also be labeled with Rh-6-(F-araNAD) (Fig S4). The results demonstrate that the mechanism-based probes can specifically label CD38 on the membrane of live cells.

For studying CD38 signaling, it is desirable if the small molecule probe does not interfere with CD38-ligand binding. We therefore tested whether labeling with Rh-6-(F-araNAD) interferes with the binding of antibodies to CD38. We treated the cells with Rh-6-(F-araNAD) first and then labeled the cells with fluorescently conjugated antibodies against CD38 (Figure 2E). Alternatively, we labeled the cells with fluorescently conjugated antibodies first and then treated the cells with Rh-6-(F-araNAD). In both cases, CD38 was labeled by both Rh-6-(F-araNAD) and antibodies (Figure 2E), suggesting that mechanism-based labeling does not interfere with antibody-CD38 interaction (Fig S5).

To optimally use these probes to study CD38 transmembrane signal, the labeling should not interfere with antibody/ligand-induced signal. We therefore tested whether labeling CD38 with Rh-6-(F-araNAD) affects the signal initiated by the CD38 agonist antibody, IB4, which causes the phosphorylation of Cbl[8] and Erk[28,29]. We labeled the CD38H cells with Rh-6-(F-araNAD) first for 30 min and then added IB4 to initiate signal. By detecting Erk and Cbl phosphorylation using Western blotting, we found that the amount of p-Erk and p-Cbl increases after addition of IB4 (Figure 2F, compare lanes 1 and 2 with lanes 3 and 4), but remains essentially the same with or without labeling with Rh-6-(F-araNAD) (Figure 2F, compare lane 1 with lane 2, and lane 3 with lane 4), demonstrating that labeling CD38 with Rh-6-(F-araNAD) does not affect antibody-induced CD38 signal.

In summary, we have developed a mechanism-based labeling method for CD38, taking advantage of the covalent trapping of CD38 by F-araNAD. Using click chemistry, 6-alkyne-F-araNAD can be conveniently conjugated to different labels, fluorescent or otherwise. Compared with GFP and antibody-based labeling, it can provide more choices of fluorophores or other labels to suit different imaging needs. The small molecule label does not interfere with the binding of or signal induced by CD38 antibodies. This labeling method thus should allow real-time monitoring of CD38 dynamics in response to ligand binding and facilitates the study of CD38-mediated signaling processes. These mechanism-based probes could in principle also

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be used to label other cell surface proteins of interest by fusing the catalytic domain of CD38 to the protein of interest, similar to the use of enzymatic biotinylation[30] and phosphopantatheinylation[31] to label cell surface proteins. Given that CD38 is expressed in certain hematological tumor cells, this labeling method may also find clinical applications. Our laboratory is currently exploring these different applications enabled by this CD38-labeling strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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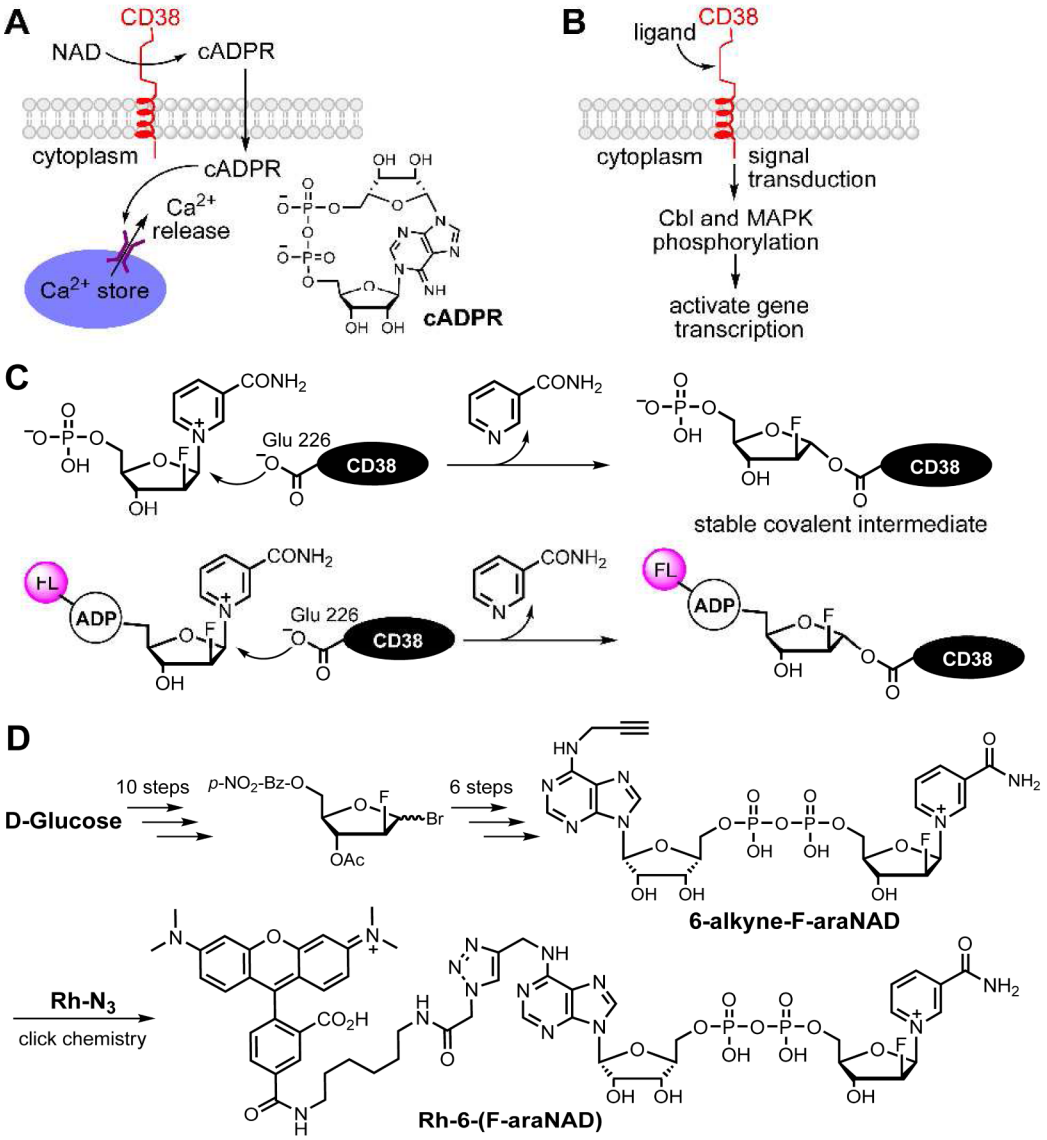

Figure 1.

(**A**) The enzymatic function of CD38 converts NAD to cADPR, which is a second messenger that can trigger Ca^{2+} release. (**B**) As a receptor, CD38 can lead to intracellular protein phosphorylation upon ligand binding. (**C**) Mechanism-based labeling strategy of CD38. (**D**) The structure and synthesis of Rh-6-(F-araNAD). Detailed synthesis is shown in Supporting Information.

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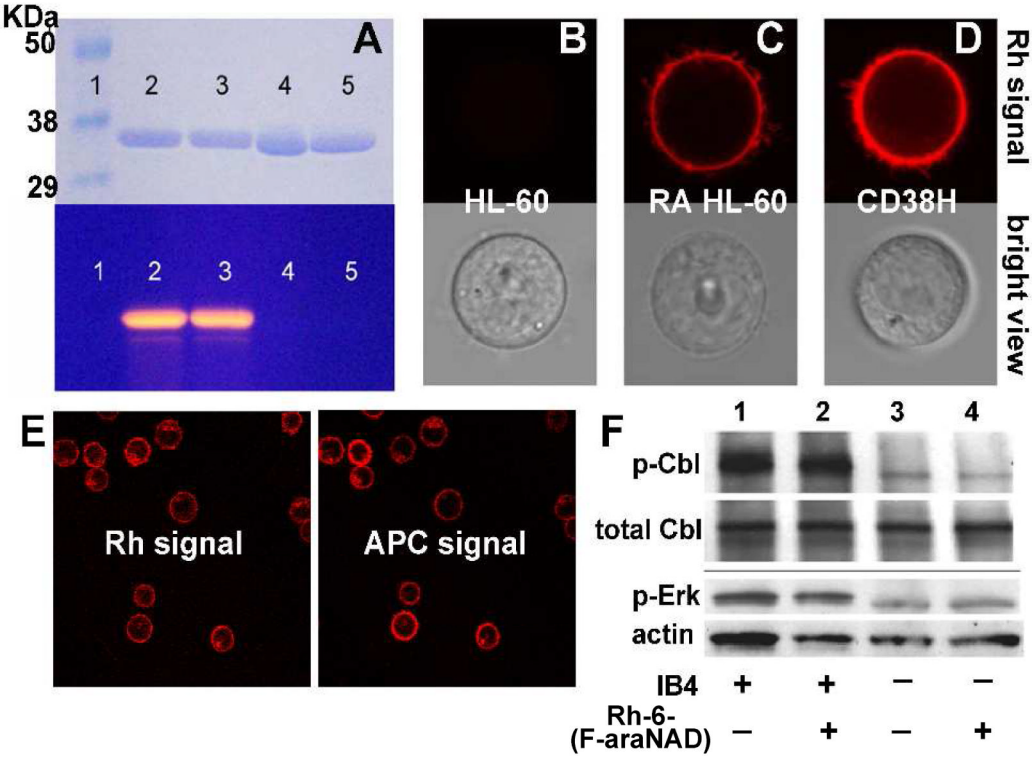

Figure 2.

(**A**) Labeling of purified CD38 proteins. Lane 1, standards; lane 2, CD38 wt with Rh-6-(F-araNAD); lane 3, CD38 wt with Rh-8-(F-araNAD); lane 4, CD38 E226D with Rh-6-(F-araNAD); lane 5, CD38 E226D with Rh-8-(F-araNAD). (**B**), (**C**), and (**D**) Confocal images of different HL-60 cells labeled with Rh-6-(F-araNAD). (**E**) Sequential labeling of RA-treated HL-60 cells with Rh-6-(F-araNAD) and then with antibody HIT2-APC shows that both can label CD38 without interfering each other. (**F**) CD38-labeling with Rh-6-(F-araNAD) does not affect the signaling initiated by the agonistic antibody IB4. Signaling was measured by detecting the phosphorylation of Cbl and Erk. The total Cbl and actin blot were used as the control to ensure equal loading in each lane.