Protein Cross-Linking

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A FlAsH-Based Cross-Linker to Study Protein Interactions in Living Cells**

Anna Rutkowska, Christian H. Haering, and Carsten Schultz**

The dynamics of protein–protein interactions play a central role in various biological processes including the function of multi-protein complexes and the regulation of signal transduction networks. Methods to sense or manipulate such interactions are therefore crucial for studying protein behavior in living cells. Protein–protein contacts can be enforced, for example, by small cell-membrane-permeable molecules which simultaneously bind to two specific protein domains.[1] While extremely useful for a range of applications, the necessity to fuse the proteins of interest to these bulky extraneous domains (usually larger than 18 kDa) often affects the proteins' activities and/or localization.[2] Moreover, the interactions generated between the additional domains are frequently irreversible not allowing the induction of transient linkages. To circumvent these restrictions, we developed a method to perform highly specific cross-linking of two proteins in living cells, each containing only a 12 amino acid peptide tag (4cys-tag). The novel cross-linking approach is based on the formation of stable, covalent complexes between a dimeric biarsenic derivative of carboxyfluorescein (xCrAsH) and two proteins containing the unique tetracysteine sequence motif CCPGCC (Figure 1a).[3] In contrast to other methods, the reaction is easily reversible by addition of membrane-permeable dithiols. As an additional feature, the protein pairs become fluorescently labeled upon binding of the cross-linker, which helps to detect and follow target proteins in real time by light microscopy.

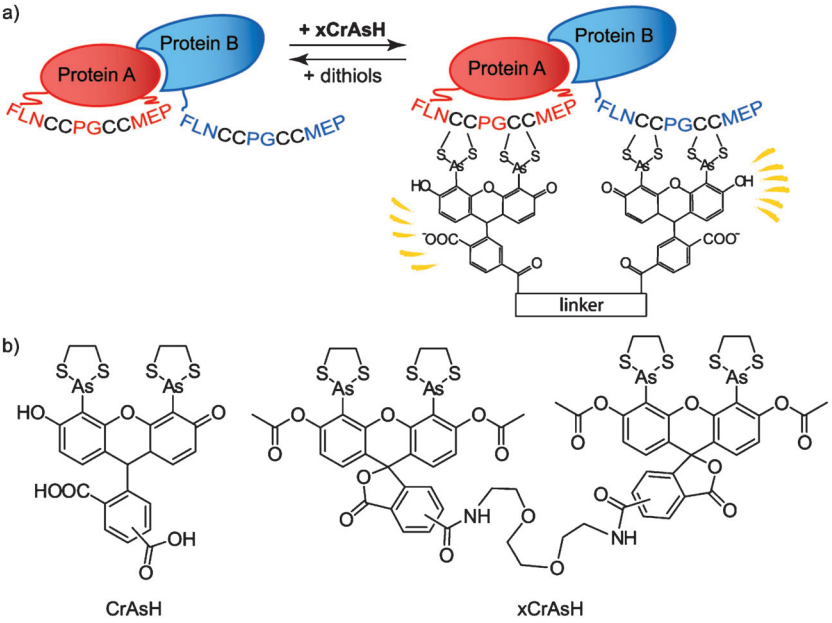

Figure 1. The cross-linker xCrAsH. a) Covalent cross-linking with xCrAsH of two interacting proteins containing internally located or terminal 4cys-tags. The cross-linked complex is fluorescent and can be released by addition of dithiols. b) Chemical structures of the CrAsH monomer (5(6)-carboxy-FlAsH) and the xCrAsH cross-linker. In contrast to CrAsH, the xCrAsH molecule is acetylated, which improves its solubility, but leads to significant reduction of its fluorogenic properties in vitro. Acetates are, however, efficiently removed by intracellular esterases. For analytical purposes, xCrAsH was also synthesized with two 5-carboxyfluorescein moieties.

Biarsenic probes in combination with a split tetracysteine tag have already been used to connect two parts of a protein structure or for monitoring the oligomeric state of proteins.[4] However, application of this approach is limited because the split motives need to be in very close proximity for sufficient cross-linking to occur. Thus, to achieve stable dimerization as well as significant flexibility of the cross-linking approach, we synthetically connected two biarsenic molecules with a flexible linker, to give xCrAsH, (Figure 1b) in an overall yield of 53% starting from carboxyfluorescein (see Supporting Information for details). In addition, the cross-linker molecule was acetylated to increase solubility during synthesis and to ensure permeability in cell applications. The single biarsenic molecule carboxy-FlAsH (CrAsH, Figure 1b) was used throughout this work as an internal control. To maintain high specificity of the cross-linking reaction, an optimized 12 amino acid sequence (FLNCCPGCCMEP, 4cys-tag) was fused to the proteins of interest.[5]

[*] Dr. A. Rutkowska, Dr. C. H. Haering, Priv.-Doz. Dr. C. Schultz
Cell Biology & Biophysics Unit, European Molecular Biology Laboratory
Meyerhofstrasse 1, 69117 Heidelberg (Germany)
E-mail: chaering@embl.de
schultz@embl.de

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As a model protein pair for cross-linking, we first chose FKBP (FK506-binding protein) and FRB (FKBP-rapamycin binding domain of mTor), two proteins that only associate with each other upon binding to the small-molecule rapamycin.[6] Based on the X-ray crystal structure of the FRB–FKBP complex,[6b] we decided to introduce the 4cys-tags at the C termini of both proteins.

We initially performed cross-linking with purified His_6-FKBP-4cys and His_6-SUMO-FRB-4cys proteins under standard conditions reported for protein labeling with biarsenic compounds.[3a] The addition of xCrAsH but not of the CrAsH monomer led to the predominant formation of a cross-linked FRB–FKBP complex in the presence of rapamycin (Figure 2a). In the absence of rapamycin, the FRB–FKBP heterodimers as well as FRB–FRB and FKBP–FKBP homodimers were detected in approximately equal stoichiometry. As expected, the cross-linked complexes were fluorescent, stable even to denaturing conditions of SDS-PAGE (polyacrylamide gel electrophoresis), and sensitive to high concentrations of dithiols (5 mM 2,3-dimercapto-1-propanol, BAL; Figure 2a and Supporting Information Figure S1). The yield of cross-linking after optimization of the assay conditions was 80% as judged by quantification using Deep Purple staining (data not shown). The optimal ratio of cross-linker to protein was between 1:1 and 2:1, which is in the range of the expected theoretical value (Supporting Information, Figure S1). Thus, these results demonstrate that xCrAsH can specifically, efficiently, and reversibly cross-link two proteins containing 4cys-tags.

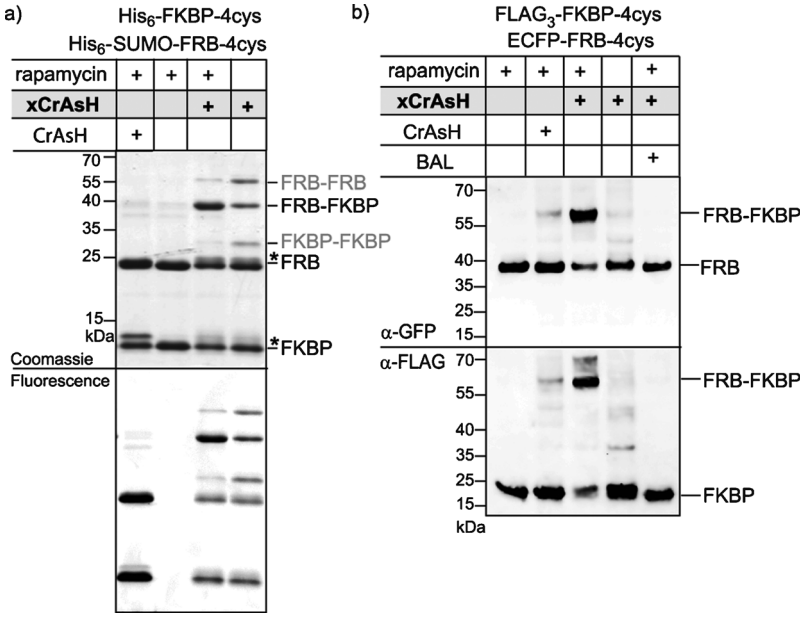

Figure 2. In vitro cross-linking of a model 4cys-tagged protein pair. a) Purified proteins (His_6-FKBP-4cys and His_6-SUMO-FRB-4cys, 15 μM each) preincubated with or without rapamycin were incubated with CrAsH (30 μM) or xCrAsH (30 μM) at 37°C for 1 h. Samples were analyzed by SDS-PAGE and Coomassie staining or in-gel fluorescence scan. Bands representing cross-linked products were visible only in the presence of xCrAsH. Complexes of protein monomers and xCrAsH or CrAsH are indicated by (*). b) Western blot of a similar cross-linking experiment using lysates from HeLa Kyoto cells transiently co-expressing $FLAG_3$-FKBP-4cys and ECFP-FRB-4cys; final concentrations: rapamycin (40 μM), xCrAsH and CrAsH (5 μM each). Cross-linked products were observed only between interacting proteins and were reverted by addition of 5 mM BAL.

We then tested cross-linking in lysates of HeLa Kyoto cells expressing $FLAG_3$-FKBP-4cys and ECFP-FRB-4cys (Figure 2b). In this system, we again achieved specific cross-linking in the presence of xCrAsH, with a yield of up to 70% as determined by quantitative western blotting (Supporting Information, Figure S1). Importantly, only the formation of rapamycin-dependent FKBP–FRB heterodimers, but not of homodimers, was detected, which demonstrates that a stable protein–protein interaction is a prerequisite for efficient xCrAsH cross-linking in cell lysates. Hence, xCrAsH can be used in cell lysates as a highly specific conditional cross-linker that does not artificially link non-interacting proteins under physiological conditions, even if the proteins contain the tetracysteine sequence.

We investigated cross-linking of 4cys-tagged proteins in living cells. Owing to the fluorogenic properties of biarsenic compounds, live-cell fluorescence microscopy is a straightforward method to test permeability and to estimate optimal concentrations of these molecules for a given cell type.[7] After incubation of HeLa or U2OS cells transiently expressing mRFP-FKBP-4cys with CrAsH or xCrAsH according to a standard method for in vivo labeling with biarsenic derivatives,[7] we observed specific fluorescent staining of cells expressing 4cys-tagged proteins, which was rapidly reverted by the addition of dithiols (Figure 3a and Supporting Information Figure S2). Thus, xCrAsH can efficiently cross the membranes of human cells and specifically binds to 4cys-tagged proteins.

To probe whether xCrAsH efficiently cross-links two tagged proteins, living U2OS (or HeLa) cells transiently expressing $FLAG_3$-FKBP-4cys and ECFP-FRB-4cys were incubated with micromolar concentrations of xCrAsH or CrAsH for 2 h in the presence or absence of rapamycin. We then measured the efficiency of cross-linking by western blotting (Figure 3b, some data not shown). As with cell lysates, the only detectable cross-linked product was the FRB–FKBP heterodimer in cells that had been incubated with rapamycin prior to cross-linker addition. Cross-linking under these conditions reached a yield of up to 50% at optimal cross-linker concentrations of 2–5 μM for U2OS and 10–20 μM for HeLa cells, and was significantly reduced when cells were subsequently treated with BAL (data not shown). xCrAsH can therefore be applied as an inducible, conditional, and reversible cross-linker in living cells.

To test whether xCrAsH can be used as a general tool to sense protein–protein interactions and to test their dependence on ligands or stimuli, we applied the cross-linker to monitor the activity state of two central heterodimeric

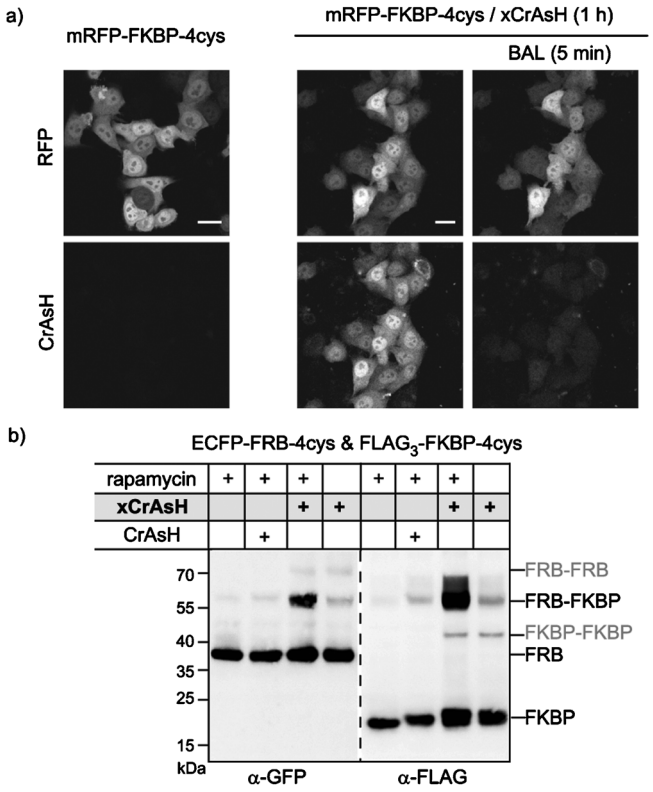

Figure 3. Cross-linking in living cells. a) Membrane permeability and specific labeling with xCrAsH: confocal images of HeLa cells transiently expressing mRFP-FKBP-4cys prior to (left) and after (middle) labeling with 1 μM xCrAsH for 1 h; CrAsH, excitation 514 nm, emission 530–600 nm. Addition of 5 mM BAL completely reversed binding of xCrAsH (right). For quantification see Supporting Information. Scale bar, 20 μm. b) Western blots after in-cell cross-linking: U2OS cells transiently co-expressing $FLAG_3$-FKBP-4cys and ECFP-FRB-4cys were preincubated with or without rapamycin (10 μM) and subsequently incubated with xCrAsH (2 μM, 2 h).

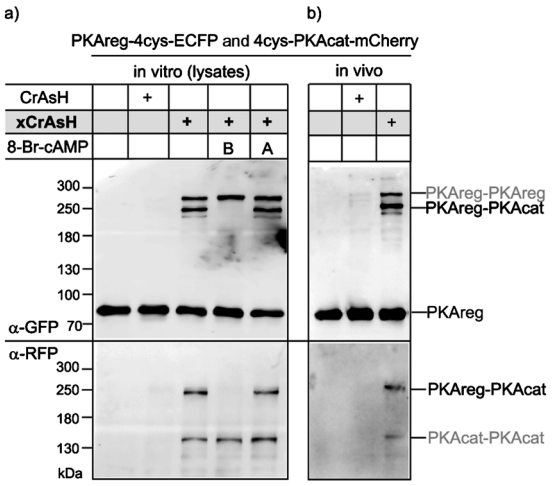

Figure 4. Probing protein–protein interactions of kinases in vitro and in living cells. a) Western blots of in vitro cross-linking experiment using lysates from U2OS cell transiently co-expressing 4cys-tag fusions of regulatory and catalytic subunits of PKA (PKAreg-4cys-ECFP and 4cys-PKAcat-mCherry) which were incubated with DMSO, CrAsH (5 μM), or xCrAsH (5 μM) at 37°C for 1 h. In some cases 8-Br-cAMP (5 mM) was added either 10 min before (B) or 50 min after (A) the addition of xCrAsH. The holoenzyme complex between PKAcat and PKAreg was detected only at low cAMP levels. b) U2OS cells incubated with CrAsH or xCrAsH (2 μM each) for 2 h at 37°C. Cells were afterwards lyzed and analyzed by western blotting. The aberrant gel running behavior of cross-links containing PKAreg is due to uncompleted denaturation of ECFP (Supporting Information, Figure S3).

kinases, namely protein kinase A (PKA) and death-associated protein kinase 1 (DAPK1). In its non-activated state, PKA is a homodimer of regulatory subunits (PKAreg) attached to two catalytic subunits (PKAcat).[8] Upon cooperative binding of four cyclic AMP (cAMP) molecules, the catalytic subunits are released and thereby activated. We would hence expect that xCrAsH is able to cross-link the catalytic subunit to the regulatory subunits before, but not after, activation of PKA by cAMP. Cross-linking reactions were performed in lysates of U2OS cells transiently expressing PKAreg-4cys-ECFP and 4cys-PKAcat-mCherry as described above. Without stimulation, heterodimers of regulatory and catalytic subunits as well as both homodimers were detected (Figure 4a and Supporting Information Figure S3). The addition of a cAMP analogue (8-Br-cAMP) to lysates prior to, but not after, the cross-linking reaction significantly reduced the amount of detected heterodimer, as predicted. In addition, PKA subunits were also efficiently cross-linked with xCrAsH in living cells (Figure 4b).

The binding of DAPK1 to its activator calmodulin depends both on the dephosphorylation of DAPK1 and on intracellular calcium levels.[9] Accordingly, we detected significant cross-linking of DAPK1-calmodulin complexes in lysates of U2OS cells transiently expressing DAPK1-4cys-ECFP (catalytic domain, 334 amino acid residues) and $FLAG_3$-4cys-calmodulin only in the presence of calcium ions (Supporting Information, Figure S4). Addition of a calcium chelating agent (ethylene glycol tetraacetic acid, EGTA) almost completely prevented protein–protein cross-linking. Thus, we successfully employed the xCrAsH cross-linker to validate the protein–protein interaction status of two kinases in cell lysates. Importantly, future optimization of the positions of the 4cys-tags may further increase the final yield of cross-linking between the described protein pairs.

In summary, we established the novel membrane-permeable chemical cross-linker xCrAsH as a tool for studying protein–protein interactions in vitro and in living cells. Three important features distinguish this technique from other approaches: reversibility (by addition of dithiols), conditionality (no forced dimerization), and the use of only a very small peptide tag (4cys-tag). In contrast to the larger domains required for alternative cross-linking methods, the 4cys-tag mimics a protein loop, which significantly reduces the likelihood of disrupting protein function, even when inserted as an internal fragment.[2c,4d,10] To increase the spectrum of applications, bifunctional cross-linkers based on the combination of one biarsenic moiety with another autoreactive functional group (e.g. benzylguanine[1d] or trimethoprim[1b] derivatives) could be developed. Such molecules will be especially important for studying multi-protein complexes, such as PKA, in which homo- and heterodimers may need to

be separated from each other. Another beneficial aspect could be the introduction of two different fluorogenic biarsenic dyes to enable more precise live cell imaging of cross-linked proteins. Finally, a photo-activatable cleavage site may be incorporated into the linker region to allow the controlled re-opening of the cross-linked interface with cellular or subcellular spatial resolution. Thus, the cross-linking technique presented herein is a first step towards the new generation of chemical inducers of dimerization, which may be generally applied to probe and manipulate protein–protein interactions in living cells.

Experimental Section

All samples from cross-linking assays were analyzed on standard SDS-PAGE using 2 × SDS sample buffer containing 100 mM tris(2-carboxyethyl)phosphine. The expression and activity of all constructs was analyzed by live cell imaging. Representative results from at least four independent experiments are presented. Detailed experimental procedures are described in the Supporting Information.

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