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Detection of S-acylated CD95 by acyl-biotin exchange

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Running title: CD95 S-acylation detection

Summary

S-acylation is the covalent addition of a fatty acid, most generally palmitate onto cysteine residues of proteins through a labile thioester linkage. The death receptor CD95 is S-palmitoylated and this post-translational modification plays a crucial role on CD95 organization in cellular membrane and thus on CD95-mediated signaling. Here, we describe the non-radioactive detection of CD95 S-acylation by acyl-biotin exchange chemistry in which a biotin is substituted for the CD95-linked fatty acid. This sensitive technique, which depends on the ability of hydroxylamine to specifically cleave the thioester linkage between fatty acids and proteins, relies on 3 chemical steps: (i) blockage of free thiols of non-modified

cysteine residues, (ii) hydroxylamine-mediated cleavage of thioester-linked fatty-acids to restore free thiols and (iii) biotinylation of free thiols with a thiol reactive biotinylation agent. Resulting biotinylated proteins can be easily purified by an avidin capture and analysed by SDS-PAGE and immunoblotting.

Key words: S-acylation, S-palmitoylation, hydroxylamine, acyl-biotin exchange

1. Introduction

Protein functions are regulated by diverse chemical modifications including lipid modifications, such as myristoylation, prenylation or S-acylation. S-acylation is the post-translational addition of a fatty acid onto the cysteine residues of proteins via a labile thioester linkage (1). Palmitic acid (16 carbons) being the predominant fatty acid linked to S-acylated proteins, this modification is generally referred as S-palmitoylation, even though other fatty acids such as oleate and stearate can be incorporated in S-acylated proteins (2). S-acylation can affect the affinity of proteins for membranes thus playing a role in membrane domains targeting, cellular trafficking and protein stability (1). In contrast to other lipidations, S-acylation is reversible and dynamically regulated by different enzymes, allowing cycles of acylation/deacylation. Indeed, the 23 members of the zDHHC protein family (characterized by the specific DHHC domain) have been described to have an acyltransferase activity while three thioesterases (PPT1, PPT2 and APT1) have been identified to date (2, 3). A wide variety of proteins are modified by S-acylation including transmembrane signaling receptors, ion channels or chaperones and increasing numbers of

DHHC/acylated substrate pairs have been identified (4). The recent development of new techniques, such as acyl-biotin exchange (ABE) to detect this modification has overcome the limitations of metabolic labelling and allowed to expand the list of known S-acylated proteins (5-7).

The importance of protein S-palmitoylation in tumor necrosis factor receptor (TNFR) superfamily signaling was reported by several scientific teams in the recent years (8-13) and our work demonstrated that this modification is necessary for an optimal cell death triggering. In particular, we focused on the CD95/CD95 ligand (CD95L) system since both proteins are constitutively S-palmitoylated (8, 10). Upon its activation by CD95L binding, the death receptor CD95 is able to initiate various signaling cascades leading either to cell death or to non-cell death functions (14). At the molecular level, CD95 S-palmitoylation occurs on the intracellular cysteine 199, close to the transmembrane domain, and is catalyzed by zDHHC7 (15). At a functional level, S-palmitoylation regulates CD95 expression and organization at the plasma membrane by allowing (i) CD95 targeting in specific membrane nanodomains enriched in cholesterol and sphingolipids (often referred as lipid rafts)(8), (ii) formation of CD95 aggregates (9) and (iii) maintenance of a proper CD95 surface expression by preventing its degradation by the lysosomes(15).

So far, S-acylation has been detected using few techniques including metabolic labeling of cultured cells with radiolabeled palmitate (^3H -palmitate) and acyl-biotin exchange (ABE). Some years ago, metabolic labeling was the most commonly used assay. Briefly, radiolabeled palmitate is incorporated in S-palmitoylated proteins, which is followed by cell lysis, immunoprecipitation of the protein of interest and resolution on SDS-PAGE for autoradiography analysis. More recently, Drisdell and Green developed a protocol called ABE

that we adapted to detect CD95 S-acylation and which relies on 3 principal steps⁽⁵⁾(Fig.1): (i) blockage of free thiols by the methylthiolating agent methylmethanethiosulfonate (MMTS), (ii) specific cleavage of thioester-linked fatty acid moieties by hydroxylamine to restore free thiols and (iii) biotinylation of the released free thiols by the thiol-reactive biotinylation agent biotin-HPDP (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide). In S-acylated proteins the fatty acids are substituted by a biotin. They can therefore be isolated by an avidin-affinity resin from the total protein lysate, resolved on a SDS-PAGE followed by immunoblotting. One should nevertheless keep in mind that ABE and metabolic labelling do not detect completely overlapping fatty acid modifications (see **Note 1**).

Both techniques present advantages and limitations. However, we decided to use the ABE protocol and adapted it to routinely analyze CD95 S-acylation for several reasons: (i) the metabolic labelling strategy presented some sensitivity limitations due to the facts that Fas expression level is quite low in the majority of the cells and that the anti-Fas immunoprecipitation step restricts the analysis to some Fas subpopulations molecules. As a consequence, several weeks or months exposure were needed to get a detectable signal on autoradiography. Comparatively, ABE is a very sensitive technique which allows the detection of endogenous S-acylated Fas by a simple western blot, the limit being here the sensitivity of the antibody used for immunoblotting detection; (ii) contrary to metabolic labelling, ABE is a non-radioactive technique which can be easily handled in a laboratory with classical security rules; (iii) while metabolic labeling is restricted to S-acylation detection in living cells, protein S-acylation status can be also assessed on tissue samples by the ABE technique.

However, important controls must be included in the ABE experiment and particular care has to be taken in order to avoid false positive hits (this will be discussed in the following protocol).

2. Materials

2.1. Cell lysate preparation

1. PBS 1X: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄.

2. Protease inhibitor cocktail: for 10 ml, combine the following components: 2 ml of 5 mg/ml Leupeptin, dissolved in water; 2 ml of 12.5 U/ml γ2-macroglobulin, dissolved in water; 1 ml of 10 mg/ml pepstatin A, dissolved in DMSO; 1 ml of 10 mg/ml chymostatin, dissolved in DMSO; and 4 ml of water. Mix well. Make small single-use aliquots of the stock solution and store at -20°C.

3. HES lysis buffer: 250 mM Hepes pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 0.1% protease inhibitor cocktail.

4. Sonicator for small sample size (eg Vibra Cell 75022).

5. Refrigerated benches centrifuges for 50 ml and Eppendorf tubes.

6. Reagents for detergent compatible protein quantification (eg DCTM protein assay, Biorad).

2.2. Acyl-biotin exchange

1. Blocking buffer (BB): 250 mM Hepes pH 7.4, 1 mM EDTA, 2.5% SDS, 20 mM methylmethanethiosulfonate (MMTS) (see **Note 2**).

2. Agitating water bath set to 50°C.
3. 100% ice-cold acetone.
4. 4HES: 50 mM Hepes pH 7.4, 1 mM EDTA, 4% SDS, 0.1% protease inhibitor cocktail.
5. HA buffer: 0.7 M Hydroxylamine (see **Note 3**), 1 mM Biotin-HPDP (see **Note 4**), 0.2% triton X-100, 0.1% protease inhibitor cocktail.
6. –HA buffer: 50 mM Hepes, 1 mM biotin-HPDP, 0.2% triton X-100, 0.1% protease inhibitor cocktail.

2.3. Avidin capture

1. Dilution Buffer (DB): 20 mM Hepes pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% protease inhibitor cocktail.
2. Washing Buffer (WB): 20 mM Hepes pH 7.4, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% protease inhibitor cocktail.
3. Neutravidin-agarose beads (see **Note 5**).
4. Laemmli buffer 4X: 240 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue.
5. β -mercaptoethanol.

2.4 SDS-PAGE and western blot.

1. 8% resolving gel buffer (for 12 ml): mix 3.2 ml of acrylamide/bis solution (29:1), 120 μ l of 10% SDS, 5.6 ml H₂O, 3 ml of Tris-HCl 1.5 M pH 8.8, 6 μ l of tetramethylethylenediamine (TEMED) and 60 μ l of 10% ammonium persulfate (APS).

2. Stacking gel buffer (for 5 ml): mix 0.66 ml of acrylamide/bis solution (29:1), 50 µl of 10% SDS, 3 ml dH₂O, 1.26 ml of Tris-HCl 0.5 M pH 6.8, 5 µl of TEMED and 25 µl of 10% APS.
3. Running buffer: 2.5 mM Tris pH 8.3, 19.2 mM glycine, 0.01% SDS.
4. PVDF membrane.
5. Transfer buffer: 2.5 mM Tris pH 8.3, 19.2 mM Glycine, 20% EtOH.
6. Tris-buffered saline tween (TBS-T) 10X: 200 mM Tris-Hcl pH 7.4, 275 mM NaCl, 1% Tween 20.
7. Blocking solution: 4% milk in TBS-T 1X.
8. First antibody dilution: 1/1000 in blocking solution. We used several antibodies to detect CD95 S-acylation: the mouse monoclonal anti-CD95 antibody B10 (Santa Cruz), the polyclonal anti-CD95 antibody C20 (Santa Cruz) and 2 rabbit monoclonal antibodies EPR520 and EPR5700 (Abcam). For the internal controls we used a rabbit polyclonal anti-fyn antibody (Santa Cruz) and a mouse monoclonal anti-GAPDH antibody (Calbiochem).
9. Secondary antibody dilution: anti-mouse or anti-rabbit secondary antibodies linked to horseradish peroxidase (HRP) are diluted to 1/10000 in blocking solution.
10. ECL reagent for chemiluminescence detection.

3. Method

3.1. Cell lysate preparation

We could detect endogenous CD95 S-acylation in both adherent and suspension cells (Fig.2a and b) (see **Note 6**). For suspension cells, centrifuge the appropriate number of cells (see

Note 7) at 300 g for 5 min at 4°C. Resuspend the cell pellet in cold PBS 1X and transfer the cells in an Eppendorf tube. Centrifuge at 300 g for 5 min at 4°C (see **Note 8**). Remove the supernatant and lyse the cell pellet with 1 ml of HES lysis buffer (see **Note 9**). In the case of adherent cells (see **Note 7**), put the plates on ice, remove the cell media, wash the cells with cold PBS 1X (see **Note 8**) and add 1 ml of lysis buffer on the plate. After scrapping the cells, recover the lysis solution and transfer in an Eppendorf tube. The lysis solution is viscous due to the presence of DNA. Sonicate the samples 2 times 10 sec (4 W) on ice to obtain a clear lysate.

Quantify your lysates for protein content using a detergent-compatible protein assay. Ideally, 3 mg of proteins per point are required.

3.2. ABE

All the following steps have to be carried out under a chemical hood.

3.2.1. Free thiol blockage: put 3 mg of proteins in a 50 ml conical tube and adjust volume to 2 ml with HES lysis buffer. Add 8 ml of blocking buffer (see **Note 10**). Incubate the samples in a waterbath at 50°C for 20 minutes under permanent agitation and frequent manual mixing.

3.2.2. Protein precipitation and MMTS removal (see **Note 11**): add 20 ml of ice-cold acetone, mix by inverting the tubes and incubate for 15 min at -20°C. Centrifuge at 2500 g for 5 min at 4°C. You obtain a visible white protein pellet. Discard the supernatant and wash the pellet with 5 ml ice-cold acetone. Centrifuge at 2500 g for 5 min at 4°C. Discard the supernatant and inverse the tubes on an absorbing paper for 2 minutes to remove the maximum of acetone. Then let the remaining acetone evaporate for 2 minutes (you can also pipet the

remaining acetone if too much is left). Add 300 μ l of 4HES and resuspend the pellet by pipetting (see **Note 12**).

3.2.3. Hydroxylamine treatment and biotinylation: split the lysate obtained in the previous step by pipetting 120 μ l in 2 dolphin Eppendorf tubes. Add 480 μ l of +HA buffer in one tube (sample with hydroxylamine) and 480 μ l of -HA buffer in the second (negative control without hydroxylamine) (See **Note 13**). Incubate for 1 hour at room temperature (RT) on a wheel.

3.2.4. Protein precipitation and biotin-HPDP removal (see **Note 14**): add 1200 μ l of ice-cold acetone in each tube, mix the tubes by inversions and incubate for 15 min at -20°C. You see the presence of a white precipitate. Centrifuge at 2500 g for 5 min at 4°C. You obtain a white visible pellet. Remove the supernatant and wash the pellet with 600 μ l of ice-cold acetone. Centrifuge at 2500 g for 5 min at 4°C. Make sure that you remove all the acetone (by pipetting the remaining acetone and let it dry 2 min) and resuspend the pellet in 120 μ l of 4HES (see **Note 12**).

3.3. Avidin capture

3.3.1. Put 100 μ l of lysate obtained in the previous step in a new dolphin tube. Add 1100 μ l of dilution buffer to reach a SDS concentration compatible with avidin capture. Centrifuge 1 min at maximum speed to remove all insoluble material. Pipet the supernatant and transfer in a new tube (see **Note 15**).

3.3.2. Pipet 90 μ l of the diluted lysate in a new Eppendorf tube to have a total lysate control before avidin immunoprecipitation and add 30 μ l of Laemmli buffer 4X (without reducing agent). Keep it at -20°C.

3.3.3. Add 45 μ l of neutravidin beads in a new dolphin tube for each sample. Add 500 μ l of dilution buffer, mix the tubes by inverting and centrifuge at 400 g for 4 min at RT (see **Note 16**). Remove the supernatant and add the remaining 1110 μ l of diluted lysate to the beads. Incubate 1 hour at RT on a wheel.

3.3.4. Centrifuge at 400 g for 4 min at 4°C (see **Note 17**). Remove the supernatant, add 1 ml of washing buffer, mix by inverting the tubes and centrifuge at 400 g for 4 min at 4°C. Repeat this washing step 3 additional times and a last one with dilution buffer.

3.3.5. Release bound proteins by reduction of the protein-biotin disulfide link with β -mercaptoethanol. Add 50 μ l of Laemmli buffer 2X containing 2% β -mercaptoethanol on the beads pellet. Incubate at 95°C for 10 min with frequent agitation, centrifuge at maximum speed for 1 min and recover the supernatant with gel loading tips (by avoiding the beads). Repeat this step with 30 μ l Laemmli buffer 2X and mix with the previously recovered supernatant (see **Note 18** and **19**).

3.4. SDS-page and western blotting.

3.4.1. Load the ABE samples on a 8% SDS-PAGE. Ideally, split your ABE sample and load half on 2 separate gels: one to detect CD95 and a second one internal S-acylated controls (see **Note 20**). Load also the lysates recovered before the avidin capture supplemented with 2% β -mercaptoethanol to detect total (S-acylated and non S-acylated) CD95.

3.4.2. Transfer on PVDF membranes.

3.4.3. Block the membranes for 1 hour at RT with blocking solution.

3.4.4. Incubate the membranes overnight with anti-CD95 (and anti-acylated protein control) antibodies at 4°C under agitation. Wash 4 times 10 min with TBS-T 1X. Incubate the membranes for 1 hour with HRP-linked secondary antibodies. Wash 4 times 10 min with TBS-T 1X. Wash a last time with dH₂O to remove salt and detergent.

3.4.5. Incubate your membranes with ECL and proceed to signal visualisation with your usual detection system (film or camera)(Fig.2).

4. Notes

1: ABE, as indicated by the name of the technique, cannot discriminate between fatty acids incorporated within the proteins but detect more generally protein S-acylation. Only additional results obtained from metabolic labelling assay allows narrowing to S-palmitoylation. However, we cannot exclude that other fatty acids are also incorporated and other approaches such as tandem mass spectrometry are necessary to profil the exact nature of the lipids attached to S-acylated proteins.

2: First prepare shortly before use 2 M dilution of MMTS in dimethylformamide (DMF) which is then used to prepare the blocking buffer.

3: Prepare a 1 M hydroxylamine solution extemporanely by diluting 3.47 mg hydroxylamine hydrochloride in 50 ml water. Adjust pH to 7.4.

4: Prepare a 4 mM stock of biotin-HPDP in DMF. Aliquot and store it at -20°C.

5: Streptavidin-agarose beads can also be used but note that Neutravidin has a higher affinity for biotin.

6: To test the specificity of the detected signal, we generated stable cell lines expressing CD95 WT or CD95 mutated on the palmitoylation site (CD95 C199V) and submitted them to ABE (Fig.2A).

7: The amount of CD95 expressing cells used per point will depend on the cell lines used and has to be adapted accordingly. As examples, $20 \cdot 10^6$ suspension T cells (Jurkat) or $15 \cdot 10^6$ colorectal SW480 cells (it represents a 80% confluent 140 mm plate) have been used.

8: This could be a pause point. For suspension cells, remove the supernatant and freeze the cell pellet by quickly throwing the Eppendorf tube in liquid nitrogen. For adherent cells, put the cell plates on ice, remove the medium, and instead of directly lysing, scrape the cells in cold PBS 1X. Centrifuge at 300 g for 5 min at 4°C, remove the supernatant and freeze the sample in liquid nitrogen. Samples can be kept in -80°C for up to one month without any obvious signal loss. Add lysis buffer directly on the frozen pellet when needed.

9: In order to optimize the CD95 proteins isolation we deliberately choose to solubilize Fas from the membranes by SDS. This choice is coherent with the following ABE steps requiring SDS for denaturation of proteins. The presence of an enriched Hepes concentration in our lysis buffer came from the fact that we also could detect Fas nitrosylation with a similar protocol (16, 17).

10: SDS-rich blocking buffer allows a complete denaturation of proteins and accessibility of MMTS to all free thiols. An incomplete blockage would result in a false positive signal for S-acylation proteins.

11: MMTS has to be totally removed as remaining traces of MMTS might compete with biotin-HPDP to thiol accessibility upon hydroxylamine action and modify the expected results.

12: The pellet can be difficult to solubilize. Do not let it dry too much before adding the 4HES buffer. Let it rehydrate in the 4HES buffer before pipetting. If the resuspension is incomplete, transfer everything in an Eppendorf tube and sonicate for 10 sec (4 W) on ice. Repeat this step if necessary.

13: Adding this control in the experiment is critical as the hydroxylamine dependence and thus the specificity of the biotin labelling has to be demonstrated by a complete absence of signal (Fig.2A and B).

14: Biotin-HPDP has to be totally removed to avoid competition with biotinylated proteins for neutravidin beads.

15: False positive hits could be easily obtained by the presence of insoluble material in your samples that would be pelleted with the beads upon centrifugation. Therefore a particular attention should be drawn when the cell lysate is recovered.

16: Pipetting the avidin beads might be sometimes delicate. Therefore, we advise before adding the cell lysate to visually verify that you have equivalent amount of beads in each tube after a quick centrifugation step.

17: After the centrifugation step, you can pipet 90 μ l of lysate and mix it with 30 μ l of Laemmli buffer 4x. This control allows checking whether your neutravidin immunoprecipitation is efficient and not saturated (enough beads to purify all the biotinylated proteins).

18: This step allows increasing the yield of recovered S-acylated proteins.

19: This could be a pause point: the samples can be kept at -80°C before loading up to one week.

20: As all S-acylated proteins are recovered in addition to CD95, others candidate proteins for S-acylation or known S-acylated proteins used as controls can be detected by specific immunoblotting. In order to compare the amount of S-acylated CD95 obtained in the different samples or conditions, and because loss of materials could be generated by the multiple steps needed, we recommend using as internal controls well-known S-acylated proteins. We used with high reproducibility the glyceraldehyde 3 phosphate dehydrogenase (GAPDH) or the tyrosine kinase Fyn. Assessing equivalent protein biotinylation by loading the protein lysates obtained before the avidin capture and blotting with streptavidin-HRP could be also used as internal control although we found this control less precise.

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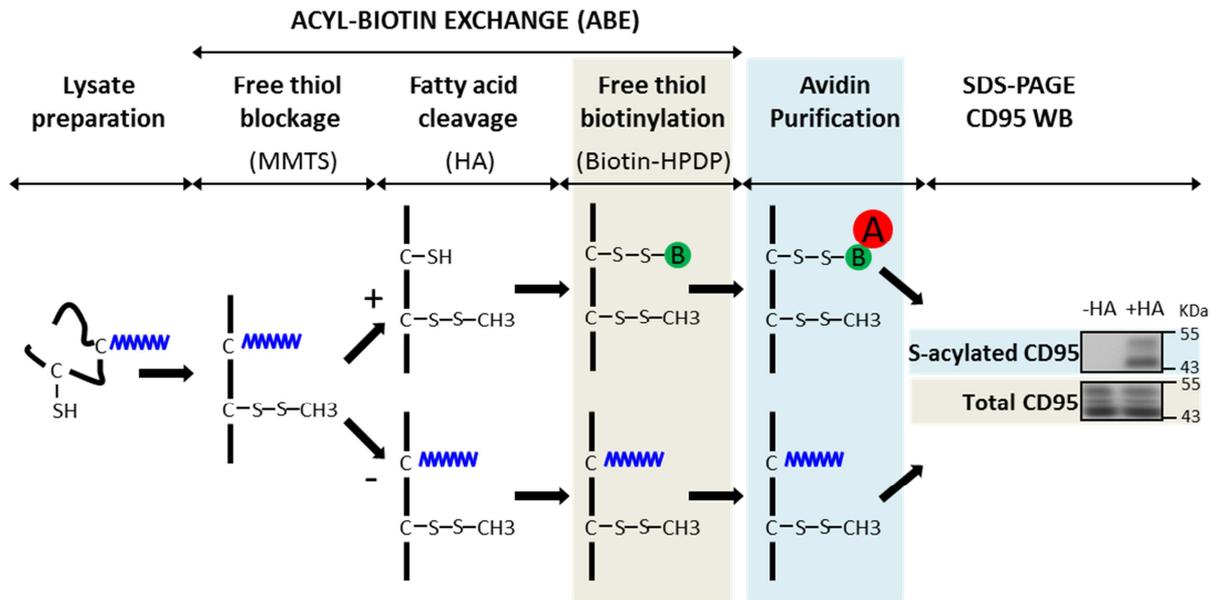
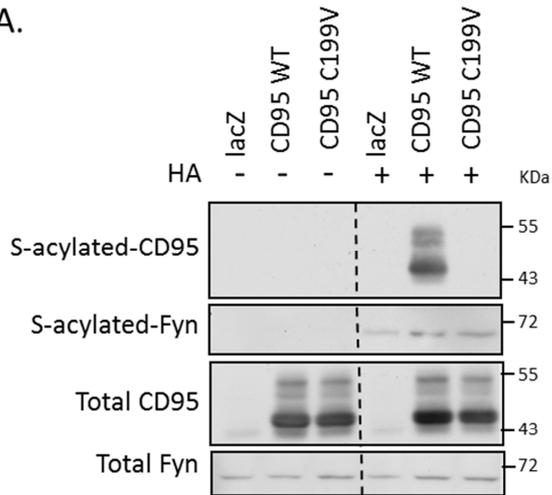


Fig. 1. Schematic representation of ABE technique. MMTS is for methylmethane thiosulfonate, HA for hydroxylamine, biotin-HPDP for N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide, B for biotin, A for Avidin and WB for western blot.

A.



B.

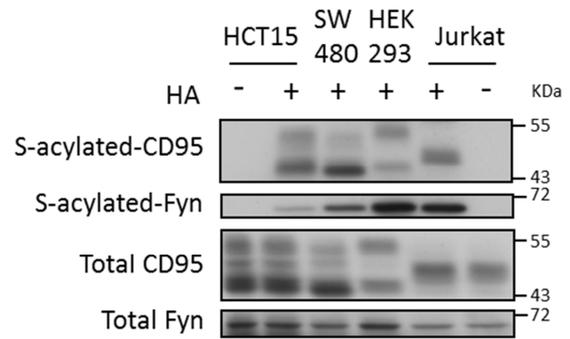


Fig.2. CD95 is S-acylated on cysteine 199. A: SW480 cells stably expressing lacZ, CD95 WT or CD95 C199V were subjected to ABE as described in the protocol. Half of the samples were exposed to hydroxylamine (+HA) and half were not (-HA). S-acylated and total CD95 (lysate recovered before avidin purification) were detected with B10 anti-Fas antibody and Fyn was used as internal S-acylated control .B: Suspension (Jurkat) and adherent (HCT15, SW480 and HEK293) cells were subjected to ABE and the endogenous level of S-acylated CD95 is shown here. S-acylated and total CD95 (lysate recovered before avidin purification) were detected with EPR520 anti-Fas antibody and Fyn was used as internal S-acylated control .