Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling

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INTRODUCTION
Cell death can be induced by a family of death receptors including Fas (TNFRSF6/CD95/Apo-1), TNFR1 [TNF (tumour necrosis factor) receptor 1; TNFRSF1A], and the two TRAIL (TNF-related apoptosis-inducing ligand) receptors DR4 (TRAIL-R1/TNFRSF10A) and DR5 (TRAIL-R2/TNFRSF10B) [1,2]. Upon activation by their specific ligands, Fas, DR4 and DR5 recruit the adaptor protein FADD [Fas-associated DD (death domain)] and caspase 8 through their cytoplasmic DD to form the DISC (death-inducing signalling complex), in which the apoptotic cascade is initiated [3–5]. All these receptors are also able to activate non-apoptotic signalling pathways [6–8]. Although the molecular events downstream of DISC formation have been intensively investigated, the initial steps engaging the death receptors to apoptotic cell fate are more elusive. In this respect, the membrane-proximal events concerning Fas engagement are better understood and a precise sequence of events has been described for Fas-mediated cell death. Several studies have highlighted the constitutive or FasL (Fas ligand)-induced raft microdomain localization of Fas [9–13]. We recently demonstrated that the S-palmitoylation of Fas in its cytoplasmic domain, which consists of the reversible addition of a palmitic acid to a cysteine, represents an essential signal targeting Fas to these rafts [14]. Moreover, palmitoylation allows Fas to associate with the cytoskeletal protein ezrin, reported to be critical for Fas-mediated cell death [15]. Indeed, the FasL-induced internalization of Fas receptor has been shown to occur in the clathrin-dependent pathway [14,16,17] and is a prerequisite for DISC formation, which occurs predominantly in endosomes [16].

However, the mechanisms regulating the initial steps of DR4- or DR5-mediated cell death are poorly understood. Several studies have described a constitutive or a raft relocation of DR4 and DR5 upon TRAIL engagement [18,19], as well as receptor-mediated endocytosis through clathrin-dependent and death receptors. We show that DR4 is palmitoylated, whereas DR5 and TNFR1 are not. Furthermore, DR4 palmitoylation is required for its raft localization and its ability to oligomerize, two essential features in TRAIL (TNF-related apoptosis-inducing ligand)-induced death signal transmission.

Key words: cell death, lipid rafts, palmitoylation, signal transduction, tumour necrosis factor receptor (TNFR).

MATERIALS AND METHODS
Antibodies and reagents
FLAG–rhTRAIL (recombinant human TRAIL) was obtained from Alexis. The DR4 and DR5 antibodies used for immunoblotting were purchased from Chemicon and Diaclove, and those used for immunoprecipitation were from Alexes. The TNFR1 antibodies used for immunoblotting and immunoprecipitation were from Santa Cruz and R&D respectively. Anti-caspase 8 was purchased from Cell Signaling, anti-ezrin from Zymed, anti-FADD from MBL, anti-caspase 3 from BD Biosciences, anti-PARP [poly(ADP-ribose) polymerase] from Biomol, and anti-Fas (C20), anti-CHC (clathrin heavy chain) and anti-caveolin-1 were from Santa Cruz.

Constructs
pcR3 DR4 C261–3S, pcR3 DR4 C261S, pcR3 DR4 C262S, pcR3 DR4 C263S, pcR3 DR4 C266S, pcR3 DR4 C274S and pcR3 DR4 C279S were obtained using the Quikchange site-directed mutagenesis kit (Qiagen) using pcR3 DR4 as a template and the primers shown in Table 1. Lentivirus expressing five different shRNA (short hairpin RNA) targeting DR5 were obtained from Sigma (MISSION® shRNA

Abbreviations used: BrP, 2-bromopalmitate; CHC, clathrin heavy chain; DD, death domain; DISC, death-inducing signalling complex; DRM, detergent-resistant microdomain; HEK cell, human embryonic kidney cell; FADD, Fas-associated DD; FasL, Fas ligand; LF, low-density fraction; 13-OP, 13-oxypalmitate; PARP, poly(ADP-ribose) polymerase; PNS, post-nuclear supernatant; shRNA, short hairpin RNA; shDR5, shRNA sequence targeting DR5; TNF, tumour necrosis factor; TNFR1, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand; rhTRAIL, recombinant human TRAIL; WT, wild-type.

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Lentiviral Transduction Particles). The chosen sequence for the shRNA was CCGGGCAGAAGA TTGAGGACCACTTCTC-GAGAAGTTGCTCCTAATCTTCTGTTTAAA. A control non-target shRNA lentiviral transduction particle expressing a scramble sequence for shRNA was also used.

Cell culture, transfection, lentiviral infection and cell lysis

HEK-293 (human embryonic kidney) cells and MDA-MB-231 human epithelial breast cancer cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% foetal calf serum. AR230 human chronic myeloid leukaemia cells were grown in RPMI supplemented with 10% foetal calf serum. HEK-293 cells were transiently transfected using the calcium phosphate technique, as previously described [22]. For palmitic acid competition with palmitic acid analogues, cells were pre-incubated with 100 μM 2-bromo palmitate (Sigma) or 300 μM 13-OP (13-oxypalmitate) for 2 h in serum-free conditions. For cholesterol depletion, cells were pre-incubated with cholesterol oxidase (Calbiochem) for 2 h in serum-free medium. HEK-293, MDA-MB-231 and AR230 cells were lysed for Western blotting with antibody against the protein of interest. Specificity of the experiment was controlled by omitting the hydroxylamine treatment.

DISC isolation

HEK-293 cells transfected with pcR3 DR4 WT (wild-type) or pcR3 DR4 C261-3S were stimulated as indicated and lysed with lysis buffer at 4 °C. The PNS (post-nuclear supernatant) obtained after centrifugation at 800 g for 10 min at 4 °C was incubated with DR4 antibody (Alexis) overnight and immunoprecipitated with Protein G–Sepharose beads (Zymed). After four washes with lysis buffer, beads were eluted with Laemmli buffer and submitted to SDS/PAGE followed by immunoblotting.

Raft isolation

Briefly, PNS from HEK-293, AR230, and MDA-MB-231 cells (2 x 10^7) were solubilized with 1% Brij 98 for 1 h at 4 °C and diluted with 2 ml buffer A containing 2 M sucrose, before being placed at the bottom of a step sucrose gradient (1.33–1.0 M) and resuspended in 1 M hydroxylamine pH 7.4 (Sigma), which specifically releases thioester-linked palmitoyl moieties and restore the modified cysteines to thiols; and (iii) which were then mixed with 0.2 M biotin–HPDP [N-(6-(biotinamido)hexyl)-3′-(2-pyridyldithio)-propionamide; Pierce] for 1 h at room temperature (25°C). Biotylated proteins were purified with neutravidin beads (Pierce), separated by SDS/PAGE and submitted to immunoblotting with antibody against the protein of interest.

RESULTS AND DISCUSSION

DR4 is palmitoylated but DR5 and TNFR1 are not

Protein palmitoylation occurs on cysteine residues located in or near the transmembrane domain. We and others have previously
Figure 1  DR4 is palmitoylated in the cytoplasmic region

(A) Sequence comparison of the membrane-proximal cytoplasmic region of human Fas, TNFR1, DR4 and DR5. The transmembrane sequence is represented in bold and cysteine residues providing putative sites for palmitoylation are highlighted in grey. (B) DR4 is palmitoylated. HEK-293 cells transiently expressing Fas, DR4, DR5 or TNFR1 were subjected to metabolic labelling with [3H]palmitate. Death receptors were immunoprecipitated with a specific antibody and analysed by both autoradiography (bottom part) and immunoblotting (using enhanced chemiluminescence). The palmitate analogues BrP and 13-OP were used to compete with [3H]palmitate and confirm the specificity of the labelling (right panel). (C) DR4 is palmitoylated on the cysteine triplet at 261–263. The four putative palmitoylation sites were mutated from cysteine to serine by site-directed mutagenesis and the palmitoylation status was assessed as in (B).

Demonstrated that human Fas is constitutively palmitoylated on Cys199 [14,25]. Sequence analysis revealed that DR4, DR5 and TNFR1 also possess one or more cysteines that are potential palmitoylation sites (Figure 1A, shaded in grey). The palmitoylation status of the receptors was assessed by incubating HEK-293 cells transiently expressing Fas, DR4, DR5 or TNFR1 with [3H]palmitate. Immunoprecipitation of the receptors followed by immunoblotting and autoradiography showed an incorporation of [3H]palmitate by DR4 (Figure 1B), which could be abolished by competition with the two palmitate analogues BrP (2-bromopalmitate; Figure 1B) and 13-OP (Figure 1B) [26], demonstrating that DR4 is palmitoylated. Under these conditions, we did not detect any palmitoylation of DR5 or TNFR1, suggesting that palmitoylation is not a general post-translational modification for DD receptors. The results of this metabolic [3H]palmitate labelling were confirmed further by using a non-radioactive method relying on acyl-biotinyl exchange chemistry, in which the S-palmitoyl thioester link is specifically cleaved by hydroxylamine exposure and substituted by a biotin moiety (Supplementary Figure S1A at http://www.BiochemJ.org/bj/419/bj4190185add.htm).

Using site-directed mutagenesis, we mutated the four putative palmitoylation sites present in DR4 and obtained four DR4 mutants: DR4 C261-3S, in which the cysteine triplet was mutated, DR4 C268S, DR4 C274S and DR4 C279S. Plasmids encoding DR4 WT and the four mutants were transfected into HEK-293 cells and the palmitoylation of the receptors was assessed. A lack of [3H]palmitate incorporation was observed only when cysteines 261 to 263 were mutated. Thus, DR4 palmitoylation occurs within this cysteine triplet (Figure 1C). In order to identify further the palmitoylated cysteine residue, we generated the single mutants C261S, C262S and C263S. However, none of the single mutations could abolish DR4 palmitoylation (Supplementary Figure S1B), thus rendering the identification of a unique amino acid as the palmitoylation site impossible. This result might reflect at least two possible situations that might be even combined: (i) several cysteines are palmitoylated at once; or (ii) only one cysteine is palmitoylated but the palmitoylation site can be interchangeable, probably due to the close proximity of the other two cysteine residues.

**DR4 palmitoylation is required for TRAIL-mediated cell death**

The function of DR4 palmitoylation was first investigated using a pharmacological approach. HEK-293 cells, which express DR4 and DR5 at a basal level, were transiently transfected with DR4 or DR5 to predominantly express DR4 or DR5 respectively. Pre-treatment with the palmitate analogue 13-OP [26] induced 60% inhibition of cell death after TRAIL engagement on DR4-transfected cells, demonstrating the important role of
Figure 2  The palmitic acid analogue 13-OP inhibits TRAIL-induced DR4-mediated cell death

(A) HEK-293 cells transiently transfected with pcR3 DR4 or pcR3 DR5 were pre-incubated with 300 μM of the palmitic acid analogue 13-OP for 2 h or left untreated. Cell death was monitored after 3 h of 250 ng/ml of FLAG–rhTRAIL plus 1 μg/ml of M2. (B) HEK-293 cells transiently expressing DR4 or DR5 were treated as in (A). Cell lysates were separated by SDS/PAGE and submitted to immunoblotting to assess PARP cleavage. (C) HEK-293, AR230 and MDA-MB-231 cells expressing shRNA targeting DR5 or a scramble (sc) sequence were pre-treated for 2 h with 300 μM 13-OP and then exposed to 250 ng/ml of FLAG–rhTRAIL plus 1 μg/ml of M2. Cell death was detected after 5 (HEK-293 and AR230) or 4 h (MDA-MB-231). Efficient DR5 knock-down was controlled by Western blot. (D) MDA-MB-231 shDR5 or MDA-MB-231 scramble cells were treated as in (C). Cells were lysed with Laemmli buffer and PARP cleavage was assayed by Western blot.

Palmitoylation in TRAIL-mediated cell death (Figure 2A). Interestingly, TRAIL-induced cell death was not affected by the 13-OP preincubation on DR5-overexpressing cells, indicating that the 13-OP inhibitory action is restricted to DR4-mediated signalling (Figure 2A). These results were confirmed at the molecular level on PARP cleavage (Figure 2B).

We then analysed the function of endogenous DR4 palmitoylation in three different cell lines which express both DR4 and DR5: the HEK-293 cell line, the chronic myeloid leukaemia cell line AR230 and the breast cancer cell line MDA-MB-231. The DR4 endogenous palmitoylation was checked using the acyl/biotin exchange technique (Supplementary Figure S1C).
We generated cells expressing DR4 as the main DD-containing TRAIL receptor by silencing DR5 in the three cell lines using lentivirus-expressing shRNA. (Figure 2C). As expected, the DR5 knockdown cells were less sensitive to TRAIL-induced cell death compared with cells expressing the scramble sequence (Figure 2C). A 2 h pre-incubation with 13-OP induced between 40 and 50% inhibition of TRAIL-induced cell death in the three cell lines expressing the scramble shRNA, indicating a major pro-apoptotic role for palmitoylation in TRAIL-mediated cell death. Interestingly, the same level of cell death inhibition was achieved by 13-OP pre-treatment when the DR5 expression was silenced, suggesting a specific contribution of DR4 palmitoylation in TRAIL-induced cell death signalling. A PARP cleavage analysis confirmed the results obtained with cell death (Figure 2D).

To investigate the specific role of DR4 palmitoylation with a genetic approach, we transfected HEK-293 shDR5 cells with an empty vector, DR4 WT and DR4 C261-3S. After verification of equal DR4 expression at the cell surface (results not shown), cell death was monitored following TRAIL stimulation (Figure 3A). Inclusion of the empty vector in the experiment allowed the role of endogenous DR4 to be distinguished. Upon TRAIL engagement, a significant induction of cell death was observed and could be clearly attributed to the transfected DR4 after 5 h of TRAIL treatment (compare pcR3 only- and pcR3 DR4 WT-transfected cells). Importantly, cell death of HEK-293 shDR5 cells transfected with pcR3 DR4 C261-3S was significantly reduced compared with DR4 WT-transfected cells, demonstrating that this palmitoylation-deficient form of DR4 is less efficient at transmitting a death signal. One can note that the mutant DR4 keeps some ability to induce cell death (compare pcR3 only- and pcR3 DR4 C261-3S-transfected cells). This decrease in cell death was confirmed at the molecular level as an inhibition of caspase 8 and PARP cleavage (Figure 3B).

We next investigated whether DISC formation was altered in presence of the palmitoylation-deficient DR4 mutant: a specific recruitment of caspase 8 and FADD to activated DR4 was observed upon TRAIL engagement in the presence of the WT receptor. In contrast, this recruitment was abolished when DR4 C261-3S was expressed (Figure 3C).

Altogether, our results demonstrate that DR4 palmitoylation is crucial for DR4 DISC formation upon TRAIL engagement and transmission of the death signal.

**DR4 palmitoylation is critical for raft localization**

Palmitoylation can be used as a signal targeting proteins to the raft microdomains [14,27]. We therefore investigated whether DR4 was located in lipid rafts. PNS from HEK-293, AR230 and MDA-MB-231 cells were solubilized with the polyethylene ether Brij 98 and subjected to ultracentrifugation through a sucrose density gradient. The DRM (detergent-resistant microdomain) rafts were then found in the LFs (low-densit fraction). Sucrose fractions were tested by immunoblotting, using caveolin and CHC as raft and non-raft markers respectively. Interestingly, both DR4 and DR5 were found almost exclusively in the rafts in all tested cell lines (Figure 4A). No change in raft localization was observed for DR4 upon TRAIL stimulation (results not shown) nor when DR5 expression was silenced (Supplementary Figure S2 at http://www.BiochemJ.org/bj/419/bj4190185add.htm). Conflicting data have been obtained concerning DR4 and DR5 raft localization in several studies. Our results are in agreement with those obtained by Song et al. [18], who observed constitutive raft localization for both receptors in non-small cell lung carcinoma. However, this is in contradiction with other studies describing constitutive non-raft localization for both DR4 and DR5 and raft recruitment only upon TRAIL stimulation [19] or drug treatment [28]. These discrepancies could be attributed to the use of different cell lines and different raft isolation techniques [29].

To address the question of the functional significance of DR4 and DR5 raft localization, we depleted the cells of cholesterol using cholesterol oxidase treatment, which disorganizes the lipid rafts. Using conditions that induce a partial loss of death receptor raft localization (Figure 4B), we observed a 60% inhibition of TRAIL-mediated cell death in both HEK-293 cells overexpressing DR4 and AR230 cells following pre-treatment with cholesterol oxidase. In contrast, staurosporine-induced apoptosis was not affected by cholesterol oxidase treatment, confirming the specificity of the treatment to TRAIL signalling (results not shown). Thus, DR4 and DR5 raft localization are
Figure 4  Palmitoylation is required to target DR4 to the rafts

(A) Brij 98-solubilized PNS from HEK-293, AR230 and MDA-MB-231 cells were submitted to low-density DRM preparation. DRMs are localized in fractions 1–4 (LF). HF denotes high-density fractions where detergent-solubilized proteins or those associated with high-density microdomains are localized. Caveolin and CHC serve as markers for LF and HF, respectively. The nine resulting sucrose fractions were immunoblotted with the indicated antibodies. (B) HEK-293 cells transfected with pcR3 DR4 and AR230 cells were preincubated for 2 h with 2 units/ml cholesterol oxidase (CO). Cell death was monitored after 3 h treatment with 250 ng/ml of FLAG–rhTRAIL plus 1 μg/ml of M2. In parallel, DR4 and DR5 DRM delocalization was confirmed by submitting HEK-293 cells treated with the indicated dose of cholesterol oxidase to DRM preparation. (C) HEK-293 cells were treated with 300 μM 13-OP for 2 h before DRM preparation as in (A). Endogenous DR4 delocalization from the raft was monitored by Western blot. (D) HEK-293 cells transfected with pcR3 DR4 WT or pcR3 DR4 C261–3S were subjected to raft preparation as in (A) and exogenous DR4 expression detected by immunoblotting.
necessary for TRAIL-mediated cell death. Similar conclusions were driven by Song et al. [18] using another cholesterol-depleting agent, methyl-\(\beta\)-cyclodextrin.

To evaluate directly the contribution of DR4 palmitoylation to DR4 raft localization, HEK-293 cells were subjected to lipid raft preparation with or without 13-OP treatment. 13-OP exposure induced a 50% delocalization of endogenous DR4 from the lipid raft, whereas DR5 localization remained unchanged, suggesting that palmitoylation represents a major signal targeting DR4 to the lipid rafts. These results were confirmed by a genetic approach using HEK-293 cells transfected with DR4 WT and DR4 C261-3S. Unlike the WT receptor, the majority of the palmitoylation-deficient DR4 was found in the non-raft fraction, clearly demonstrating that palmitoylation is necessary for raft localization (Figure 4D). However, an alternative signal targeting DR4 to the rafts appears to exist, since a small proportion of the DR4 C261-3S could still be found in the raft fraction. Interestingly, DR5 (Figure 4A) and TNFR1 (results not shown) are also directed to the rafts by a palmitoylation-independent mechanism, since these two receptors are associated to lipid rafts despite the fact that they are not palmitoylated (Figure 1B). For TNFR1, the DD itself was reported to be necessary and sufficient for this localization [30].

### Palmitoylation influences DR4 oligomerization

Using non-reducing conditions for raft sample preparation, we observed that, in addition to causing relocalization of the monomer form of DR4 into non-raft fractions (Figure 4D), the mutation also affected higher oligomer forms (results not shown). Immunoblotting of non-reducing total lysates with DR4 antibody showed very different migration patterns for DR4 WT and DR4 C261-3S. Three different oligomer bands were observed for the WT receptor (Figure 5A), whereas only one band (n°1) was predominant in the C261-3S DR4 sample. Bands 2 and 3 were only visible after long exposure of the film. Thus not only the non-raft localization, but also this altered oligomerization capacity of the palmitoylation mutant, could explain its reduced ability to induce cell death.

Moreover, although DR4 WT presented a doublet band for the monomer, only the lower one was observed for the palmitoylation-deficient mutant, suggesting that the upper band represents palmitoylated DR4, which can be detected in the absence of reducing agents. Thus only some of DR4 WT is palmitoylated.

DR4 is able to form homo-oligomers comprising several DR4 molecules, but also hetero-oligomers with other TRAIL receptors such as DR5 [4,19]. In order to investigate the composition of oligomers formed by DR4 WT and DR4 C261-3S, we performed co-immunoprecipitations with anti-DR4 or anti-DR5 antibodies of HEK-293 cells transfected with the indicated plasmids (Figure 5B) and immunoblotted them using anti-DR4 antibody. Although the oligomerization pattern obtained after the DR4 immunoprecipitation was similar to the one obtained from total lysates, only band n°2 contained DR5 and no DR4 C261-3S/DR5 interaction was observed.

All together, the present results pointed out the essential role of the post-translational modification of the death receptor DR4 by palmitoylation, which we show is required for the localization of the receptor in lipid rafts, and therefore for an efficient transmission of the cell death signal. Interestingly, we reported that DR4 palmitoylation is not only required for its localization to lipid rafts but also for its homo-oligomerization and for its association with DR5 (hetero-oligomerization). Future studies will be needed to investigate the exact functional correlation of lipid raft association of the TRAIL receptors, the formation of the DR4–DR4 or DR4–DR5 complexes and their death-inducing capacities.

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SUPPLEMENTARY ONLINE DATA

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Figure S1 Determination of palmitoylation using the acyl/biotin exchange technique

(A) HEK-293 cells were transiently transfected with pcR3 Fas, pcR3 DR4, pcR3 DR5 or pcR3 TNFR1 and subjected to an acyl/biotinyl exchange technique. After immunoprecipitation with neutravidin beads, samples were subjected to SDS/PAGE and immunoblotted with anti Fas, DR4, DR5 and TNFR1 antibody respectively. Controls without hydroxylamine treatment were included to assess the specificity of the detection. (B) HEK-293 cells transiently expressing the indicated wild-type or mutant DR4 were subjected to an acyl/biotinyl exchange technique as in (A). Lanes 1 and 2 are from another exposure of the same experiment to compare the similar level of transfection. (C) Acyl/biotinyl exchange experiment on HEK-293, AR230 and MDA-MB-231 cells performed as in (A) to detect endogenous DR4 palmitoylation.

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Figure S2  DRM isolation on AR230 and MDA-MB-231 cells expressing shDR5 or shscramble

Samples were treated as in Figure 4 of the main text.