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Analysis of human tumour necrosis factor receptor 1 dominant-negative mutants reveals a major region controlling cell surface expression

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Abstract Tumour necrosis factor receptor 1 (TNFR1) plays a critical role in host defence and inflammation. We have identified a membrane proximal region (aa 218–324) of TNFR1 that restricts surface expression. This was prompted by comparing the dominant-negative properties of a C-terminal truncation of TNFR1 with a point mutant that prevents signalling. C-terminal truncation (aa 218–426) generates a better dominant-negative TNFR1 mutant than inactivation of the death domain by point mutation. The increased dominant-negative activity correlates with increased cell surface expression. The membrane proximal region is the most important region of the receptor for restricting expression.

Keywords: Tumour necrosis factor receptor 1; Nuclear factor-κB; Structure–function; Dominant-negative receptor; Surface expression

1. Introduction

Tumour necrosis factor receptor 1 (TNFR1/CD120a) is a death-domain (DD) containing receptor and one of two receptors for the cytokine TNFα [1]. It is the high affinity receptor for soluble TNFα and is predominantly responsible for the effects of TNFα even when the other receptor, TNFR2 (CD120b), is present [2–4]. TNFα signalling plays a critical role in host defence, proliferation, apoptosis, inflammation and differentiation [5].

The extracellular domain of TNFR1 is involved in the formation of a pre-ligand oligomeric receptor complex and in TNF binding. TNF binding to TNFR1 activates downstream signalling pathways via a number of adapter proteins, which bind to the cytoplasmic domain of TNFR1 [6]. The DD (aa 320–415) [7] (Fig. 1A) is able to recruit the adapter proteins, TNFR DD protein [8], Fas-associated DD protein [9], and the serine/threonine kinase, receptor RIP [10]. The DD is essential for the activation of the nuclear factor-κB (NF-κB) pathway and activation of the apoptotic caspase cascade. The C-terminus also contains sequences which bind the factor associated with neutral sphingomyelinase (FAN, aa 310–318) [11] and the adapter protein, growth receptor bound 2 (Grb2, aa 237–240) [12]. The FAN-binding sequence alone is required for the activation of the neutral sphingomyelinase [11] and, together with the Grb2-interacting sequence, for activation of the c-Raf-1 kinase [12].

This study initially investigated TNFR1 dominant negatives for use as a research tool. Mutants were generated by changing specific amino acids or by deletion of the adapter protein-binding domains, and their signalling and dominant-negative properties were assessed by transient transfection of the Eli-BL B-cell line. We show that deletion of the TNFR1 cytoplasmic domain generates the most efficient TNFR1 dominant negative. Furthermore, this mutant displayed an extremely high cell surface expression when compared to wild type (Wt) or point mutated TNFR1. A region negatively regulating TNFR1 cell surface expression was mapped to the membrane proximal region of the cytoplasmic domain.

2. Materials and methods

2.1. Cell lines

Eli-BL [13] and DG75 [14] B-cell lymphoma cell lines were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and 200 U/ml penicillin and 200 mg/ml streptomycin antibiotics and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Basal production of TNFα was determined for both cell lines by ELISA (Duoset kit, R&D Systems) and was found to be <20 pg/ml. This was in agreement with previous values determined for Eli-BL and other BL lines [20].

2.2. Plasmids

The vector pTARGET-TNFR1 Wt (provided by Dr. Michael Lenardo, National Institutes of Health, Bethesda, USA [15]) was used to amplify the entire human TNFR1 coding region and shorter sequences, which were cloned into the pcDNA3.1–V5/His directional TOPO cloning vector (Invitrogen). The common forward primer 5′ CACCATGGGCCT- CCACCGTGCC 3′ was used with the following reverse primers: 5′ TCTGAGAAGACTGGGCGCGG 3′, 5′ AATGGAGTAGAGCTTGGAC 3′, 5′ ATCAGTGTCTAGGCTCTG 3′, 5′ GCCCAGCAGGTCCATGTCG 3′, 5′ AAGCG- CCTCCTCGATGTCC 3′ and 5′ ACTGGGCGCGGGGGCGAG 3′ to generate the TNFR1 Wt, Δ218, Δ325, Δ403, Δ413 and Δ424 constructs, respectively. The TNFR1 ΔFAN, AA and ΔAA mutants were generated from TNFR1 Wt, using the Quikchange site-directed mutagenesis kit (Stratagene), using the following primers; 5′ CCCAACCCCCTTCCACAGAGCCTAG 3′ and its reverse complement (ΔFAN), and 5′CGCTGGA AGGAAGCCGTGGCGCGCCTAGGGCTGAGC 3′ and its reverse complement (AA). The 3Enh.κB-ConALuc reporter (3Enh-Luc) contains three tandem repeats of the NF-κB binding sites from the Igκ promoter upstream of a minimal conalbumin promoter

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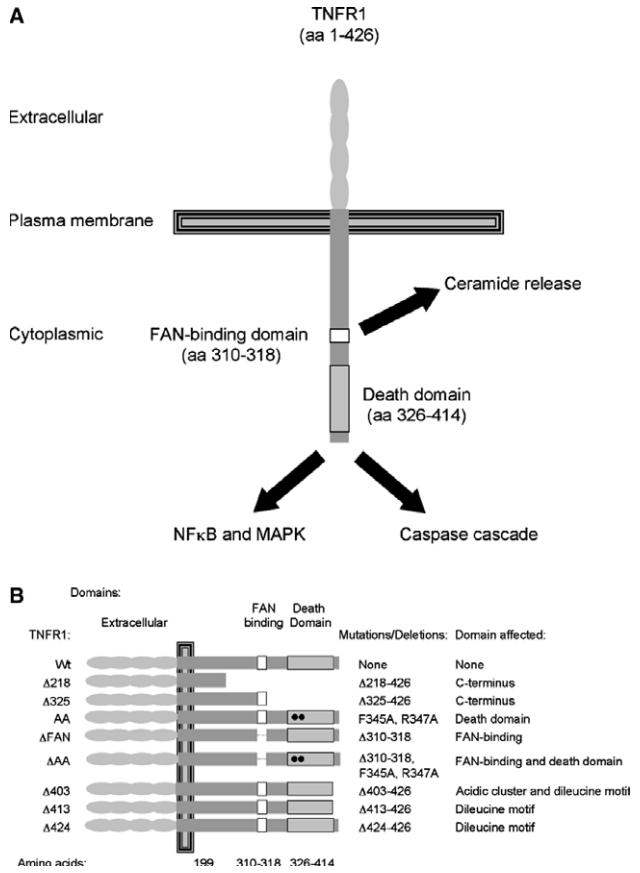

Fig. 1. (A) Schematic representation of TNFR1 domains and signalling pathways. (B) Panel of TNFR1 mutants utilised, showing the mutations and affected domains.

[16]. The enhanced green fluorescent protein expression vector (EGFP), pEGFP-N1, was purchased from Clontech.

2.3. Gene transfection and luciferase reporter assay

Up to 10^7 cells in 0.5 ml RPMI medium buffered with 100 mM HEPES, pH 7.2, were transfected using a Bio-Rad Genepulser II electroporator (280 V/950 μF for Eli-BL; 270 V/950 μF for DG75). The cells were seeded in fresh growth medium. Transfection efficiency was typically 5–20% for Eli-BL and 40–50% for DG75. Transfected cells were either mock-stimulated with growth medium or stimulated with 10 ng/ml TNFα (purchased from R&D Systems), 16 h post-transfection. Luciferase activity was measured 8 h after stimulation with TNFα, exactly as described previously [17].

2.4. Detection of cell surface TNFR1 expression (CD120a) by flow cytometry

Cell surface expression of TNFR1 in the transfected cell population was assayed by flow cytometry of immunofluorescent staining. Cells, harvested 24 h after transfection, were stained with anti-human TNFR1 mouse monoclonal antibody (Ab-1, Oncogene research products) or an IgG_1 isotype control antibody, followed by a rabbit anti-mouse phycoerytherin (PE)-conjugated secondary Fab fragment (Dako). Transfected cells were marked by the expression of cotransfected EGFP-N1 plasmid and were gated for analysis of TNFR1 staining.

3. Results

3.1. Generation of human TNFR1 mutants and characterisation of their signalling ability

A panel of human TNFR1 mutants was generated in the pcDNA3.1 vector (Fig. 1B). They were designed to delete large portions of TNFR1 cytoplasmic C-terminus or to disrupt specific C-terminal domains. NF-κB activation by the TNFR1 mutants was assessed by luciferase reporter assay of transiently transfected Eli-BL cells (Fig. 2A), a B-cell line, which had a low endogenous response to TNFα, compared with other cell lines tested (data not shown). Eli-BL cells were co-transfected with each TNFR1 construct or with empty vector and NF-κB luciferase reporter plasmid, split into two, and 16 h later, one half stimulated with TNFα and the other mock stimulated. After 8 h incubation, the cells were harvested and assayed for luciferase activity.

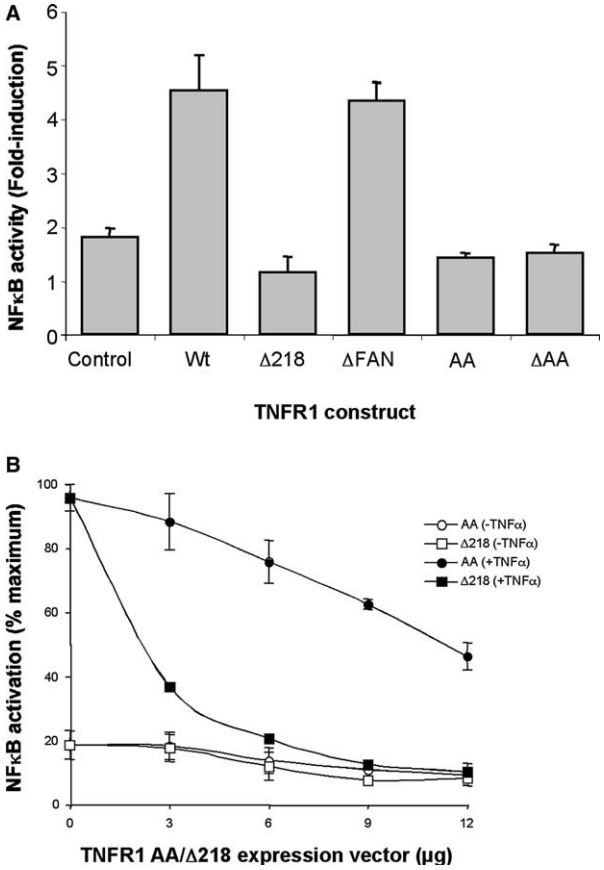

Fig. 2. (A) NF-κB activation by TNFR1 adapter protein-binding domain mutants. Eli-BL cells were transiently transfected with 3 μg NF-κB luciferase reporter, 2 μg EGFP-N1 and 6 μg of each TNFR1 construct. Transfections were split into two. Sixteen hours post-transfection, one half was mock stimulated and the other stimulated with TNFα (10 ng/ml). After 8 h, luciferase activity was assayed. The results are shown as fold-induction of NF-κB activity, calculated by division of the raw value from the TNFα-stimulated well by the mock stimulated well, for each transfection. The results are mean, and standard error of at least three independent experiments. (B) TNFR1 Δ218 is a more efficient dominant negative than TNFR1 AA. Eli-BL cells were transiently transfected with 3 μg NF-κB luciferase reporter, 2 μg EGFP-N1, 6 μg TNFR1 wild type, and increasing amounts of TNFR1 AA (circles) or Δ218 (squares). (Total DNA amount kept constant at 21 μg, by addition of appropriate amounts of empty vector.) Transfections were split into two and 16 h post-transfection one half was mock stimulated (white-filled) and the other stimulated with 10 ng/ml TNFα (black-filled). After 8 h, luciferase activity was assayed. The results are shown as % maximal luciferase activity within each experiment and are mean and standard error of three independent experiments.

TNFα stimulation of the control transfection resulted in a 2-fold increase in NF-κB activity over that detected in the unstimulated control transfection (Fig 2A). Transfection of TNFR1 Wt resulted in a 4.5-fold induction of NF-κB activation, in response to TNFα. Removal or mutation of the death domain (Δ218, AA and ΔAA) resulted in no activation of NF-κB. Removal of the FAN-binding domain (ΔFAN) had no effect on induction of NF-κB, compared to Wt. The TNFR1 mutants Δ218 and AA, were selected to test as dominant-negative mutants of TNFR1 signalling, because of their inability to activate NF-κB.

3.2. Ability of TNFR1 mutants to inhibit wt TNFR1 NF-κB signalling

The effectiveness of the TNFR1 Δ218 and AA constructs to inhibit wild type receptor NF-κB signalling was compared, revealing significant differences between the two mutants (Fig. 2B). Fig. 2B shows that co-transfection of the TNFR1Δ218 deletion mutant inhibits wild type TNFR1 signalling much more effectively than the point mutant, TNFR1 AA. In the absence of TNFα, increasing amounts of both constructs had little effect on the basal level of NF-κB activation. Addition of TNFα to cells transfected with Wt TNFR1 alone resulted in a 5-fold increase in NF-κB activity. Transfection with increasing amounts of TNFR1 Δ218 resulted in a marked decrease of TNFα-induced NF-κB activation. 6 μg of TNFR1 Δ218 was sufficient to reduce the NF-κB activity to basal levels. Transfection with increasing amounts of TNFR1 AA resulted in a less marked decrease of TNFα-induced NF-κB activation. 12 μg of TNFR1 AA was not sufficient to reduce the NF-κB activity to the basal level and resulted in only a 45% reduction in the maximal activity.

In addition, 12 μg of either TNFR1 Δ218 or AA was unable to inhibit NF-κB activation by co-transfection of a constitutively active viral TNFR1 mimic encoded by Epstein–Barr virus, the latent membrane protein-1 (data not shown). This demonstrated that the dominant-negative effect of the TNFR1 mutants was specific to TNFα-induced signalling. These data demonstrate that a specific TNFR1 dominant negative can be generated by mutation of the adapter protein-binding domains. However, deletion of the TNFR1 C-terminus generated a more efficient dominant negative than disruption of the DD by the introduction of point mutations.

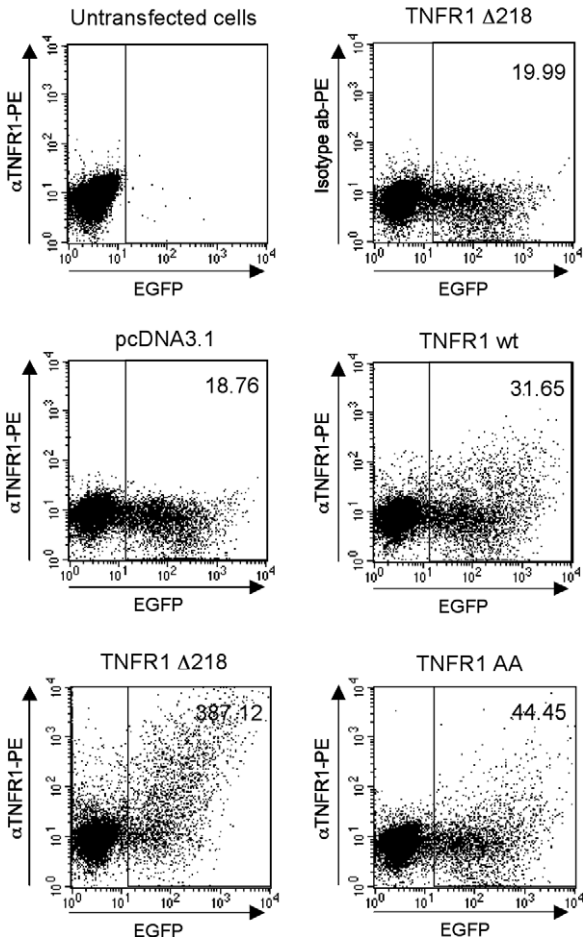

Fig. 3. Expression of TNFR1 constructs. DG75 cells were transfected with 1 μg EGFP-N1 and 10 μg empty vector, pcDNA3.1 or an expression vector for TNFR1 Wt, TNFR1 Δ218 or TNFR1 AA. Expression was investigated by flow cytometry with an anti-TNFR1 antibody or irrelevant IgG_1 antibody and a PE-conjugated secondary Fab fragments. Results are shown as dot plots against anti-TNFR1-PE or isotype antibody-PE fluorescence against EGFP fluorescence and are representatives of three independent experiments. The m.f.i of staining for the transfected cell gate is shown in the top-right hand corner of the dot plot. Untransfected cells and TNFR1 Δ218 transfected cells stained with isotype antibody are shown for comparison.

3.3. TNFR1 Δ218 cell surface expression levels are much higher than TNFR1 Wt or AA

We sought to identify the mechanism for the difference between the TNFR1 Δ218 and TNFR1 AA dominant negatives. To test the hypothesis that differences in expression levels were responsible, expression studies were performed in two cell lines. One cell line was Eli-BL, the same line used for the analysis of NF-κB activation. We also used a highly transfectable DG75 B-cell line that was unsuitable for the signalling assays because it contains a high level of constitutive NF-κB activity but which allowed a more sensitive analysis of TNFR1 expression. The expression of TNFR1 constructs on the cell surface of viable transfected cells was measured by flow cytometry (Fig. 3 and Table 1) of cells stained with a mouse monoclonal antibody against human TNFR1 and a PE-conjugated secondary antibody. Transfected cells were indicated by the expression of GFP. Control non-transfected cells, cells transfected with empty vector and GFP and cells transfected with TNFR1 Δ218 stained with an isotype control antibody were also analysed in parallel.

The average mean fluorescence intensities and standard errors of TNFR1 staining of the transfected cell population are shown in Table 1. There was a difference between the levels of transfected TNFR1 expressed at the cell surface in both cell types (Table 1). However, more expression of TNFR1 Wt could be seen in DG75 cells. The basal level of TNFR1 staining (pcDNA3.1) was the same as that obtained using an irrelevant isotype control antibody. This indicates that both cell types express either no TNFR1 or levels below the limit of detection. In both cell types, TNFR1 Δ218 was extremely highly expressed, whereas TNFR1 Wt and AA expression was lower generally, but still detectable. Higher levels of TNFR1 Δ218 were also detected by fluorescence microscopy of fixed and stained cells (data not shown). A proportion of TNFR1 construct transfected cells showed no increase in TNFR1 staining above basal levels. This may reflect TNFR1 levels

Table 1
Cell surface expression of TNFR1 constructs in transfected DG75 and Eli-BL cells

Construct	TNFR1 staining in DG75 cells (average m.f.i. ± S.E.M.)	TNFR1 staining in Eli-BL cells (average m.f.i. ± S.E.M.)
pcDNA3.1	18 ± 1	13 ± 1
TNFR1 Wt	32 ± 2	24 ± 2
TNFR1 Δ218	325 ± 34	508 ± 215
TNFR1 Δ325	84 ± 13	ND
TNFR1 AA	46 ± 1	30 ± 3
TNFR1 ΔFAN	50 ± 2	ND
TNFR1 ΔAA	32 ± 5	ND
TNFR1 Δ403	72 ± 6	ND
TNFR1 Δ413	38 ± 4	ND
TNFR1 Δ424	33 ± 2	ND

The average mean fluorescence intensity of TNFR1 staining of the transfected (GFP positive) population for pcDNA3.1empty vector and TNFR1 transfections from three independent experiments. TNFR1 Δ218 transfected cells were also stained with an isotype IgG_1 antibody as a control (average m.f.i. 19 ± 3 and 10 ± 1 for DG75 and Eli-BL, respectively). ND, not done.

below the sensitivity of our assay, or that in these cells the majority of TNFR1 was localised internally. There were no differences in cell viability between the transfections. The inhibition of Wt TNFR1 signalling observed in Fig. 2B correlates with the increased cell surface expression of the C-terminal truncation mutant TNFR1 Δ218, relative to the point mutant TNFR1 AA observed in Fig. 3.

3.4. Negative regulation of cell surface expression by a membrane proximal region of the TNFR1 cytoplasmic C-terminus

A previous study identified a region of the DD responsible for lipid raft localisation (aa 405 and 412) [18]. This region of TNFR1 is also involved in localisation to the *trans*-Golgi (aa 403–426), and contains a putative acidic cluster (aa 406–410) and a dileucine motif (aa 424–425) [19], sequences known to affect protein localisation. To examine whether this region influenced TNFR1 cell surface expression, we constructed a further three deletion mutants (TNFR1 Δ403, Δ413 and Δ424). One deletion (Δ403) removed this entire region including both the acidic cluster and the dileucine motif. The other deletions (Δ413 and Δ424) removed only the dileucine motif (see Fig. 1).

First, the ability of TNFR1 Δ403, Δ413 and Δ424 to activate NF-κB was assessed by luciferase reporter assay in transiently transfected Eli-BL cells (Fig. 4). Addition of TNFα to the control transfection resulted in approximately a 1.5-fold activation of NF-κB. The TNFR1 Wt transfection resulted in a 6-fold activation of NF-κB. As expected, transfection of the C-terminal deletion mutant TNFR1 Δ218 responded in a similar way to control transfection. Deletion of the entire region involved in lipid raft localisation (Δ403) abolished NF-κB activation. Shorter deletions (Δ413 and Δ424), which retained the acidic cluster (aa 406–410), were Wt in their activation of NF-κB.

Expression studies were performed, as before, in the DG75 cells. These cells allow a higher expression of TNFR1 Wt and so permitted more careful analysis of expression of these subtle mutations. DG75 cells were transiently transfected with all TNFR1 constructs, including another deletion mutant (Δ325), which was unable to activate NF-κB (data not shown). The average mean fluorescence intensities and standard errors of TNFR1 staining of the transfected cell population are shown

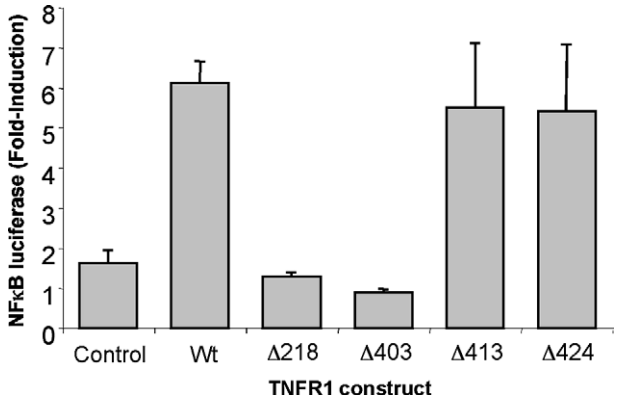

Fig. 4. NF-κB activation by the TNFR1 Δ403, Δ413 and Δ424 deletion mutants. Eli-BL cells were transiently transfected with 3 μg NF-κB luciferase reporter, 2 μg EGFP-N1and 6 μg of each construct. Transfections were split into two and 16 h post-transfection one half was mock stimulated and the other stimulated with 10 ng/ml TNFα. After 8 h, luciferase activity was assayed. The results are shown as fold-induction of NF-κB activity, calculated by division of the raw value from the TNFα-stimulated well by the mock stimulated well, for each transfection. The results are means and standard error of three independent experiments.

in Table 1. TNFR1 Wt expression resulted in a 1.8-fold increase in the m.f.i. of the transfected (GFP positive) cell population. TNFR1 Δ218 expression was 22-fold higher than that of TNFR1 Wt. Mutation or deletion of the FAN-binding and death domains had little effect on the cell surface expression of TNFR1. Deletion of short sections of the TNFR1 extreme C-terminus (Δ413 and Δ424) also had little effect of TNFR1 cell surface expression. Deletion of the recently identified *trans*-Golgi localisation sequence (Δ403) and a shorter deletion of the TNFR1 C-terminus (Δ325) resulted in a small but significant increase in TNFR1 cell surface expression (approximately 4 to 5-fold increase on Wt levels). A region negatively regulating TNFR1 cell surface expression therefore maps to a sequence between amino acids 218 and 324.

4. Discussion

TNFR1 signalling plays a critical role in innate immune function and inflammation and has also shown to be important for the growth of B-cell lymphoma cell lines [5,20]. This study investigated the generation of an efficient dominant-negative TNFR1 mutant by disruption of adapter protein-binding domains. However, the removal of the full cytoplasmic tail of TNFR1 (Δ218) was a more efficient inhibitor of Wt signalling, compared with the point mutant (AA). C-terminal mutants had been described previously [7,21] and have been shown to inhibit TNF-induced apoptosis. They were unable to induce apoptosis in stable transfectants, in response to TNFα, and they had some degree of dominant-negative ability because the levels of apoptosis were reduced relative to control transfectants. The relative dominant-negative efficiency and expression of TNFR1 point mutants versus deletion mutants has not previously been determined.

The different ability of TNFR1 mutants to inhibit Wt signalling correlated with TNFR1 surface expression. Removal of a *trans*-Golgi localisation sequence [19] did result in a signifi-

cant increase in cell surface expression, although not to the extremely high levels observed for Δ218. Interestingly, aa 405–413 within this same sequence was shown by another group to be important for the localisation of TNFR1 to lipid rafts [18]. The equivalent mutants in our study indicate a requirement for this sequence, which contains an acidic cluster, in NF-κB signalling. This is interesting in light of a recent report, which showed that lipid rafts were essential for NF-κB signalling by TNFR1 [22]. These results demonstrate that the amino acid sequence responsible for the TNFR1 lipid raft localisation is also essential for NF-κB activation.

Further expression studies showed that another TNFR1 mutant (Δ325) with amino acids 325–426 deleted was expressed at similar levels to Δ403. Therefore, a region of the TNFR1 cytoplasmic C-terminus between amino acids 218 and 324 negatively regulates TNFR1 cell surface expression. This region of TNFR1 is known to contain a number of phosphorylation sites [23] and phosphorylation of these sites can relocalise TNFR1 [24]. It also contains a sequence that interacts with the SH3 domain of Grb2 (aa 237–240) [12]. Interestingly, the interaction of Grb2 with epidermal growth factor receptor has a role in its internalisation by clathrin-coated pits [25,26].

TNFR1 expression plays a major role in restricting TNFR1 function and the outcome of its signalling in response to TNF. This report identifies an important region, between amino acids 218 and 324, with a major role in regulating TNFR1 cell surface expression. Removal of this sequence greatly increased TNFR1 cell surface expression and the dominant negative efficiency of the resulting mutant.

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