A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in 'reverse signalling'

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We have identified a putative signalling feature of the cytoplasmic domains of the tumour necrosis factor (TNF) family members based on available amino acid sequence data. A casein kinase I (CKI) consensus sequence is conserved in the cytoplasmic domain of six of 15 members of the type II integral membrane TNF ligand family. We examined the phosphorylation state of transmembrane tumour necrosis factor- α (mTNF) with [³²P]orthophosphate labelling and *in vitro* kinase assays, in lipopolysaccharide-stimulated RAW264.7 cells. A dimeric form of the type I soluble TNF receptor (sTNFR) was found to dephosphorylate mTNF. This effect could be prevented by treatment with phosphatase inhibitors. Recombinant CKI was able to phosphorylate mTNF that had been dephosphorylated by sTNFR ligation *in vivo*, and this was less effective if phosphatase inhibitors had been used to prevent mTNF dephosphorylation. A mutated form of mTNF, lacking the CKI recognition site, cannot be phosphorylated by the enzyme. Binding of sTNFR to mTNF induced an increase in intracellular calcium levels in RAW264.7 cells, implying the presence of an associated signalling pathway. We predict that this CKI motif is phosphorylated in other TNF ligand members, and that it represents a new insight into the mechanism of 'reverse signalling' in this cytokine family.

Keywords: casein kinase I/macrophage/phosphorylation/receptor/reverse signalling/TNF- α

Introduction

Members of the tumour necrosis factor (TNF) ligand superfamily (with the exception of lymphotoxin α) are type II integral membrane proteins, and possess the unusual feature of a large, and highly evolutionarily conserved, leader sequence which spans the cell membrane and presents a small N-terminal cytoplasmic portion. As yet, no function has been ascribed to this intracellular domain of the protein. Deletion mutation analysis of the cytoplasmic domain of transmembrane TNF (mTNF) has revealed that this region does not affect proper insertion of mTNF into the cell membrane, nor does it affect proteolytic processing

to release the soluble 17 kDa TNF (sTNF) molecule (Utsumi *et al.*, 1995). However, the high sequence conservation suggested to us that it could somehow be involved in the recently characterized phenomenon of 'reverse signalling', whereby the ligand acts like a receptor. In this process, cellular signals are transduced by the membrane-bound ligand, upon binding of soluble or membrane-bound receptors belonging to the TNF receptor superfamily (Smith *et al.*, 1994; Bazzoni and Beutler, 1996). This phenomenon has been described recently for various members of the TNF ligand family (Cayabyab *et al.*, 1994; Pollok *et al.*, 1994; Stuber *et al.*, 1995; van Essen *et al.*, 1995; Wiley *et al.*, 1996). The functional importance of reverse signalling remains to be established, though it has been shown to play a role in a range of different immune processes, including cytokine production (Wiley *et al.*, 1996), co-stimulation of T-cell activation (Cayabyab *et al.*, 1994; van Essen *et al.*, 1995; Wiley *et al.*, 1996) and proper formation of germinal centres (van Essen *et al.*, 1995).

Phosphorylation of human mTNF has been reported previously (Pocsik *et al.*, 1995), and shown to be restricted to serine residues. Based on the reported DNA sequences, there are a total of four conserved serine residues within the cytoplasmic domain of mTNF in 14 species of eutherian mammals (see Discussion). However, only two of these serine residues, -STES- present on the N-terminus of the cytoplasmic domain distant from the cell membrane, are present in one species of marsupial TNF that has been cloned (Wedlock *et al.*, 1996). Taking into account the extent of divergence between marsupial mammals and eutherian mammals (~130 million years) (Delbridge *et al.*, 1997; Janke *et al.*, 1997), it seems likely that the evolutionary pressure to conserve these residues is related to a critical functional role, and these residues are the primary candidates for phosphorylation. Double-spaced serine residues [-S(P)XXS*-, where the central two amino acids are unimportant, S(P) indicates a phosphoserine and S* indicates the target residue] loosely define a phosphorylation site for casein kinase I (CKI) (Kennelly and Krebs, 1991). The presence of acidic residues N-terminal to the phosphoserine is also favoured with a CKI site (Tuazon and Traugh, 1991). We have compared all currently available predicted amino acid sequences of TNF ligand family members, both between species and between family members. A CKI motif (-SXXS-) is present in six out of 15 of the family members, and is conserved amongst all species cloned (see Discussion). Acidic residues are present N-terminally within two residues of a serine in Fas ligand, TNF- α , CD27 ligand and human 4-1BB ligand, and within eight residues of a serine in CD30 ligand, CD40 ligand and mouse 4-1BB ligand.

In this study, we have investigated the possible involvement of sTNFR in regulation of the phosphorylation of

Fig. 1. The 26 kDa TNF is phosphorylated in LPS-stimulated RAW264.7 cells. Phosphorylation status was examined by [32 P]H $_3$ PO $_4$ labelling of cells for the final 2.5 h of the 4 h incubation period. Cells were either unstimulated (lane A) or stimulated with LPS in the absence (lanes B and C) or presence (lanes D and E) of BB1101. In some groups, sTNFR was added for the final 15 min of incubation (lanes C and E). Total cellular 26 kDa TNF was then immunoprecipitated from cell lysates with anti-TNF antibody. A representative gel of four independent experiments is shown. The summary of the densitometric analysis is shown in the histogram above the gel. The values represent the relative phosphorylation signal normalized by expressing each densitometry value as a percentage of the sum of the values of the 26 kDa TNF band in all lanes (mean $\pm$ SEM).

mTNF and intracellular signalling, and show that CKI is likely to be one of the kinases responsible for phosphorylating mTNF.

Results

Phosphorylation of mTNF is modulated by sTNFR in a phosphatase-dependent fashion

Using orthophosphate labelling, it was found that 26 kDa TNF, on lipopolysaccharide (LPS)-stimulated RAW264.7 cells, is phosphorylated (Figure 1). This phosphorylation was restricted to the intracellular portion of the molecule as the 17 kDa sTNF molecule was not phosphorylated (data not shown). The compound BB1101 afforded greater sensitivity of detection of 26 kDa TNF. This drug is a hydroxamic acid-based inhibitor of TNF processing with selective inhibitory activity towards the release of sTNF (Gearing *et al.*, 1995). In the absence of this drug, the detection of 32 P-labelled, total cellular 26 kDa TNF was very faint, bordering on the detection limits of the phosphorimager (Figure 1, lane B). Consequently there was a large degree of variability associated with densitometry conducted on these bands. Addition of sTNFR, for the last 15 min of the labelling period, to RAW264.7 cells treated with LPS plus BB1101 induced a significant ($\sim 30\%$, $P = 0.0286$) dephosphorylation of the signal of total cellular 26 kDa TNF (Figure 1, lanes D and E). Our previous observations indicate that this effect is not due to reduced efficiency of immunoprecipitation, caused by sTNFR blocking anti-TNF antibody recognized epitopes, nor can sTNFR induce a change in the expression of total cellular 26 kDa TNF (A.D.Watts, N.H.Hunt, M.Madigan and G.Chaudhri, submitted). However, sTNFR ligation was unable to induce a similar reduction in signal in cells that had not been treated with BB1101. This may simply reflect the limitations of detection of mTNF in cells without BB1101, or possibly the incompatibility of continual processing of mTNF with stable engagement of sTNFR. Another feature of the results (Figure 1) was the presence

Fig. 2. Effect of phosphatase inhibitors on the phosphorylation status of 26 kDa TNF. RAW264.7 cells were either unstimulated (lane A) or stimulated with LPS for 4 h in the absence (lane B) or presence (lanes C–F) of BB1101. [32 P]H $_3$ PO $_4$ was added for the final 2.5 h of this period. Cells were then incubated on ice for 30 min with (lanes D and F) or without (lanes A–C and E) phosphatase inhibitors, calyculin A and sodium fluoride (indicated by *Pi*). In some groups, sTNFR was added for a further 15 min (lanes E and F). Total cellular 26 kDa TNF (lanes A–D) or mTNF (lanes E and F) was then immunoprecipitated from cell lysates, as described in Materials and methods. A representative gel of four independent experiments and associated summary histogram with densitometry values (normalized as described in Figure 1) is shown.

of higher molecular weight bands that probably represent glycosylated isoforms of the 26 kDa TNF protein, as reported by us (Watts *et al.*, 1997) and others (Jue *et al.*, 1990). Our data suggest that both the glycosylated and the non-glycosylated forms of 26 kDa TNF are phosphorylated.

Next we investigated the action of phosphatase inhibitors on the phosphorylation state of mTNF from cells that had been treated with sTNFR. Cells were exposed to the serine/threonine phosphatase inhibitors, calyculin A and sodium fluoride, in an attempt to prevent sTNFR-induced dephosphorylation. Incubation of stimulated RAW264.7 cells with these agents caused no significant difference in the phosphorylation of total cellular 26 kDa TNF (Figure 2, lanes C and D). However, in cultures where sTNFR was added, the dephosphorylation of the mTNF fraction was prevented by the phosphatase inhibitors (Figure 2, lanes E and F).

Casein kinase I phosphorylates mTNF

A CKI site was found to be conserved in all 15 species of mammalian 26 kDa TNF, close to the N-terminus of the cytoplasmic domain (as outlined in the Discussion). To determine whether CKI is responsible for phosphorylating mTNF, a selective *in vivo* and *in vitro* inhibitor of CKI, the isoquinolinesulfonamide compound CKI-7 (Chijiwa *et al.*, 1989), was used to treat RAW264.7 cells. The compound was added to the cells at the time of stimulation with LPS, and was present for the entire 4 h incubation, including the orthophosphate labelling period. Phosphorylation of mTNF was almost completely abolished by 150 μ M CKI-7 (Figure 3, lanes E and F), an effect which could not be explained by a reduction in TNF synthesis (data not shown). CKI-7 also reduced the phosphorylation signal of total cellular 26 kDa TNF by 82% (Figure 3, lanes C and D). To demonstrate further the role of CKI in phosphorylation of mTNF, an *Escherichia coli*-derived

Fig. 3. Effect of the CKI inhibitor, CKI-7, on the phosphorylation state of TNF. RAW264.7 cells were either unstimulated (lane A) or stimulated with LPS for 4 h in the absence (lane B) or presence (lanes C–F) of BB1101. In some groups (lanes D and F), CKI-7 was also added at the same time. [^{32}P]H $_3$ PO $_4$ was added for the final 2.5 h of this period. As in Figures 1 and 2, the phosphorylation status was examined by [^{32}P]H $_3$ PO $_4$ labelling of cells for the final 2.5 h of the incubation period. Total cellular 26 kDa TNF (lanes A–D) or mTNF (lanes E and F) was then immunoprecipitated from cell lysates, as described in Materials and methods. A representative gel of three independent experiments and associated summary histogram with densitometric values (normalized as described in Figure 1) is shown.

Fig. 4. Recombinant CKI is able to phosphorylate mTNF *in vitro*. RAW264.7 cells were stimulated with LPS for 4 h in the presence of BB1101 and in the absence (lanes A, B and D) or the presence (lane C) of phosphatase inhibitors, calyculin A and sodium fluoride. sTNFR was added for the final 15 min of incubation to dephosphorylate mTNF *in vivo*, before it was immunoprecipitated. This was then used as a substrate in a kinase reaction, described in Materials and methods, catalysed by recombinant CKI (lanes B–D). The CKI inhibitor, CKI-7, was also added to this reaction in some groups (lane D). No substrate was added to the samples in lane A. A representative gel of four independent experiments and associated summary histogram with densitometric values (normalized as described in Figure 1) is shown.

recombinant rat CKI (Graves *et al.*, 1993) was used to study the effectiveness of immunoprecipitated mTNF as a substrate. We found that recombinant CKI was capable of phosphorylating immunoprecipitated mTNF (Figure 4, lane B) that had been dephosphorylated by treatment with sTNFR for 15 min at 37°C in the same manner as described in Figure 2. If the phosphatase inhibitors sodium fluoride and calyculin A were added at the same time as sTNFR *in vivo*, then phosphorylation *in vitro* was reduced by 2.6-fold ($P = 0.0571$; Figure 4, lane C), indicating that the recombinant CKI-dependent phosphorylation is specific to the site naturally phosphorylated in intact cells.

Fig. 5. The putative CKI phosphorylation site is required for the phosphorylation of mTNF by recombinant CKI. (A) Targeting of the candidate CKI phosphorylation site. Mutagenesis was carried out on a human TNF genomic clone capable of expressing a 26 kDa protein deficient in processing ($\Delta 1$ –12 mutation). The region containing the four amino acids of the CKI site (–STES–) in the wild-type (W) sequence was substituted with a different one of similar length (M). The mutated region is underlined. The mutant and wild-type cDNA were constructed in the mammalian expression vector pCDNA3 under control of the constitutive CMV promoter. The position of the adjacent transmembrane region is indicated. (B) Mutant mTNF is not phosphorylated by CKI *in vitro*. Human 293-T cells were transiently transfected, with vector only (lane D) or with vector containing either the mutant (lane A) or wild-type (lane B and C) form of 26 kDa TNF. At 24 h post-transfection, cells were incubated with sTNFR to dephosphorylate mTNF *in vivo*. Cells were then lysed and immunoprecipitated with TNF1 monoclonal antibody. This was then used as a substrate in a kinase reaction, described in Materials and methods, catalysed by recombinant CKI (lanes A, B and D). No CKI was added to the samples in lane C. The top arrow corresponds to the molecular weight of recombinant CKI- δ used in the kinase assay. It is known that this isoform of the enzyme is able to autophosphorylate (Rivers *et al.*, 1998).

If the CKI was omitted from the reaction buffer (lane A) or CKI-7 was added to the reaction (lane D), there was little or no phosphorylation. Recombinant CKI also phosphorylated mTNF that had been immunoprecipitated from cells not treated with BB1101, and this was similarly less efficient if phosphatase inhibitors were added *in vivo* (data not shown).

To demonstrate the importance of the N-terminal –STES– site in phosphorylation by CKI, a mutant form of mTNF lacking this site was expressed in 293-T cells, and tested for its ability to act as a substrate for recombinant CKI. The mutant and wild-type constructs were based on a non-cleavable ($\Delta 1$ –12) human TNF clone described previously (Grell *et al.*, 1995), and cloned into the expression vector pCDNA3. The expected protein sequences of the cytoplasmic domain of these two TNF constructs are shown in Figure 5A. Following immunoprecipitation of the total cellular 26 kDa fraction with TNF1 monoclonal antibody, an *in vitro* kinase assay was carried out, as described above for endogenous TNF from murine RAW264.7 cells. The phosphorimage is shown in Figure 5B. It was found that the mutant form (M) was not phosphorylated detectably by recombinant CKI (Figure 5B, lane A), whereas the wild-type (W) molecule was phosphorylated very effectively in the same assay (Figure 5B, lane B). The kinase assay of the wild-type molecule in the absence of CKI revealed a little residual

Fig. 6. sTNFR is able to induce an increase in intracellular calcium in RAW264.7 cells expressing mTNF. The intracellular calcium level in cells was monitored using Fura-2, as described in Materials and methods. Cells stimulated for 4 h with LPS in the presence of BB1101 to express mTNF (□) showed stable baseline levels of intracellular calcium. Upon addition of sTNFR (■), an increase in intracellular calcium was observed. In contrast, sTNFR had no effect on the intracellular calcium levels in cells stimulated in the presence of 150 μ M CKI-7 (▲). Also, if stimulated cells were pre-treated with the phosphatase inhibitors, calyculin A (50 nM) and sodium fluoride (50 mM), for 20 min prior to the addition of sTNFR, no increase was observed (○). In control experiments, boiled (100°C, 10 min) sTNFR was added to stimulated cells (●). Values represent the mean calibrated calcium concentrations from, typically, six individual cells in each independent experiment ($n = 4$, mean $\pm$ SEM).

phosphorylation (Figure 5B, lane C), indicating co-immunoprecipitation of endogenous kinase activity. Cells transfected with vector only (Figure 5B, lane D) showed little or no signal. To check that the cells were transfected with approximately equal efficiency, they were co-transfected with a construct constitutively expressing luciferase protein. Measurement of the luciferase activity in an aliquot of the cells was determined by a luminometer, and the values (expressed as relative light units, RLU) were 265 360 RLU (cells corresponding to lane A), 259 590 RLU (cells corresponding to lane B), 265 560 RLU (cells corresponding to lane C) and 160 560 RLU (cells corresponding to lane D). Furthermore, the protein expression of both the CKI site mutant and wild-type constructs in transfected 293-T cells was analysed by [35 S]methionine labelling followed by immunoprecipitation plus SDS-PAGE. It was found that the mutant and wild-type molecules were expressed as a 26 kDa protein at similar levels (data not shown).

sTNFR-induced changes in intracellular calcium levels

In order to link the ligation of sTNFR to mTNF with a known intracellular signalling pathway, we examined the effects of this on the intracellular levels of calcium. To study the effect of sTNFR on mTNF-expressing cells, LPS-stimulated RAW264.7 cells were treated with BB1101 as described for the experiments above. The use of BB1101 for this kind of experiment eliminates complications that could arise through sTNFR neutralizing soluble 17 kDa TNF in the extracellular space and thereby down-regulating possible autocrine effects of the cytokine. Measurements of intracellular calcium levels were taken at 5 min intervals following the addition of sTNFR or controls (Figure 6). Treatment with sTNFR induced a marked increase in intracellular calcium levels (~250% over con-

trols), that reached a plateau at 10 min post-stimulation. This increase was not observed with heat-inactivated (100°C for 10 min) sTNFR, eliminating the possibility of endotoxin contamination.

To investigate the possibility that agents that modulate mTNF phosphorylation also affect the sTNFR-induced increase in calcium, phosphatase inhibitors and CKI-7 were tested. Cells stimulated in the presence of CKI-7 did not respond to sTNFR with an increase in intracellular calcium. Similarly, when phosphatase inhibitors, calyculin A and sodium fluoride, were added to cells, 20 min prior to the addition of sTNFR, no increase in intracellular calcium was observed. The sTNFR-induced increase in calcium was sensitive to lanthanum (10 μ M, added just before sTNFR) and did not occur in the absence of extracellular calcium (data not shown).

Discussion

The mechanism of the newly observed phenomenon of 'reverse signalling' in TNF ligand family members has yet to be unravelled. Here, we provide the first evidence that these cytokines have the potential to function in a manner reminiscent of cell-bound receptors regulated by CKI phosphorylation.

We have examined the phosphorylation state of mTNF, a typical type II integral membrane protein (Kriegler *et al.*, 1988; Perez *et al.*, 1990), with features characteristic of the TNF ligand family (Bazzoni and Beutler, 1996). The use of a specific inhibitor of TNF processing, the peptide-hydroxamate class (Gearing *et al.*, 1994; McGeehan *et al.*, 1994; Mohler *et al.*, 1994) compound BB1101, causes a build-up of mTNF that is 5- to 8-fold greater than in untreated cells (A.D.Watts, N.H.Hunt, M.Madigan and G.Chaudhri, submitted). This resulted in sufficient mTNF levels to examine the phosphorylation state of this molecule in murine macrophages. Upon stimulation, TNF is normally expressed in this cell type, and is therefore accompanied by the full set of molecular machinery associated with its function and regulation. The inhibition of mTNF processing in RAW264.7 cells, due to BB1101 treatment, also mimics physiological situations in which mTNF may not be processed for a variety of reasons, for example at low doses of LPS (Chaudhri, 1997), and presents the sTNFR or cell-bound TNFR with a more stable molecular target conducive to signal transduction. The finding that mouse mTNF is phosphorylated (Figure 1) also lends evolutionary significance to this modification, since human mTNF is known to be phosphorylated (Pocsik *et al.*, 1995).

We found that treatment of cells for 15 min with dimeric sTNFR leads to the dephosphorylation of total cellular 26 kDa TNF (Figure 2). This dephosphorylation must represent the fraction of the TNF present on the cell surface (i.e. mTNF), as the high molecular weight sTNFR could not enter the cells to interact with the intracellular 26 kDa TNF. The sTNFR-induced dephosphorylation of the 26 kDa mTNF molecule could be blocked by the phosphatase inhibitors sodium fluoride and calyculin A (Figure 2). This provides compelling evidence that sTNFRs are dephosphorylating mTNF, and that this is through a mechanism dependent on one or more serine/threonine phosphatases. Our next objective was to identify the

enzyme responsible for the initial phosphorylation of mTNF.

CKI is a widely expressed, cyclic nucleotide-independent serine/threonine kinase, located in the nucleus and cytosol, and associated with membranes (Tuazon and Traugh, 1991). CKI has been shown to phosphorylate various proteins from these cellular fractions. Examples of membrane proteins that are CKI substrates include the insulin receptor (Tuazon *et al.*, 1985) and the p75 TNF receptor (Beyaert *et al.*, 1995; Darnay *et al.*, 1997). The single most important specificity determinant for CKI has been shown to be a phosphoserine located two amino acids before the target serine or threonine [$-S(P)XXS^*/T^*-$] (Flotow and Roach, 1989; Flotow *et al.*, 1990). Peptide studies by Meggio *et al.* (1992) have shown that phosphothreonine is a comparable substitute for phosphoserine as a CKI specificity determinant, whilst phosphotyrosine is poor in this respect. It has been noted that many natural substrates phosphorylated by CKI contain acidic residues N-terminal to the CKI phosphorylated serine residue (Tuazon and Traugh, 1991). This property has been characterized in fine detail by peptide studies (Meggio *et al.*, 1992), and the consensus D_nXXS^* has been defined (where $n = 3, 4 > n = 2 > n = 1$ in terms of substrate effectiveness). However, Flotow and Roach (1991) found that even the optimal CKI substrate containing acidic residues was a poor substitute for a substrate containing phosphoserine. There is great diversity within different CKI substrates, which might be explained to some extent by the presence of multiple CKI isoforms (Zhang *et al.*, 1996). This diversity remains a complication when trying to identify potential CKI sites in proteins, and there is no consensus sequence for CKI entered in any of the popular protein database search programs. In the case of the TNF ligand family members, we have chosen the candidate CKI sites manually, based on the presence of the consensus $-SXXS-$, and the likelihood that these are CKI sites is reinforced by acidic residues present N-terminal to the second serine.

Using three approaches, we have provided both *in vivo* and *in vitro* evidence to support the notion that mTNF is an effective CKI substrate, and that CKI is the hitherto unknown physiological serine/threonine kinase responsible for phosphorylating mTNF. In the first case, we used a selective inhibitor of CKI to inhibit mTNF phosphorylation *in vivo* (Figure 3). Secondly, we were able to show that recombinant CKI phosphorylates immunoprecipitated mTNF at the site that is naturally phosphorylated *in vivo* (Figure 4). Finally, using a mutant form of human mTNF, we showed that the region containing the CKI site, which is conserved across all mammalian species in the cytoplasmic domain of TNF, is essential for the *in vitro* phosphorylation by recombinant CKI (Figure 5). A precedent for a phosphorylation-regulated signalling mechanism involving CKI is the type II transmembrane TNF receptor, where inhibition of endogenous CKI increases the apoptotic signal delivered by TNF (Beyaert *et al.*, 1995). It is intriguing that both components of the bi-directional TNF ligand-receptor pair appear to have some degree of similarity in their signalling mechanism. We propose that the identification of this group of CKI sites, shown in Figure 7, represents the first insight into the mechanism of cytokine 'reverse signalling', and predict that they are

functional in other members of the TNF ligand family. Further work directed at determining the phosphorylation state of other TNF ligand family members may help to elucidate this unusual pathway, especially as there is growing evidence for the existence of biological responses as a result of reverse signalling in many of these members. In fact, only the members which have thus far been demonstrated to show reverse signalling contain the CKI motif. This list includes Fas ligand (Suzuki and Fink, 1998), which contains a 'double' CKI site. In addition, the presence of the CKI motif [$-S(P)XXS-$] implies the involvement of a different kinase, other than CKI, that is important for phosphorylation of the first serine residue, to confer specificity of the site for CKI.

It was found that sTNFR induced an ~250% elevation of intracellular calcium levels, which was maximal ~10 min post-stimulation (Figure 6). Similar increases in intracellular calcium concentration, in macrophages/monocytes, have been associated with various biological responses such as interferon- γ -induced antigenic expression of HLA-DR, Fc γ R and CR3 (Klein *et al.*, 1990). In addition, Higuchi *et al.* (1997) found that cross-linking mTNF, expressed on HTLV-1-infected T-cells, with anti-TNF antibody resulted in a gradual increase in intracellular calcium levels over a 25 min period. Our experiments with lanthanum and calcium-free medium indicate that the increase in intracellular calcium occurs through an influx of this anion from extracellular sources. The fact that two different types of pharmacological agents (phosphatase inhibitors and a CKI inhibitor) that modulate the phosphorylation of mTNF also abrogate the increase in calcium (Figure 6), suggests a link between the mTNF phosphorylation state and sTNFR-induced calcium signalling. This provides the first direct evidence of a signalling pathway operating through sTNFR engagement of mTNF. Previously, biological roles for sTNFR have been defined purely on the assumption that sTNFR is a neutralizing agent and carrier for sTNF. We suggest that the biological effects of sTNFR may be more significant than previously appreciated, and that sTNFR is capable of acting like a ligand through ligation to mTNF.

The significance of reverse signalling in TNF ligand family members in the context of the immune system at a holistic level is not generally understood, though an exception is CD40 ligand, for which there is good *in vivo* evidence for a role in germinal centre formation (van Essen *et al.*, 1995). Given the elevated levels of soluble receptors of the TNF receptor superfamily, and in particular sTNFR, in many pathological conditions (Aderka *et al.*, 1991; Girardin *et al.*, 1992; Godfried *et al.*, 1993; Molyneux *et al.*, 1993; van Deuren, 1994; Falini *et al.*, 1995; Kalinkovich *et al.*, 1995), it might be the case that reverse signalling acts to modulate the immune response to disease through regulation of cytokine expression.

The presence of a conserved CKI site in the cytoplasmic domain of the TNF ligand family members is notable, given the very poor homology of the rest of the cytoplasmic domain between members. It is likely that the site may serve an important function. The work presented here strongly suggests that CKI is involved in the process of reverse signalling through mTNF. We predict that this will be the case in other members of the TNF family. The evolutionary implications for this medically important

Fig. 7. (A) Alignment of the amino acid sequences for the entire cytoplasmic region of mTNF across 14 species of eutherian mammals and one species of marsupial mammal. Sequences were obtained from the Swiss protein database and have been predicted from cDNA sequences. Conserved serine residues are indicated by black shading, and acidic residues are highlighted with grey shading. (B) Comparison of putative CKI motifs within the cytoplasmic domain of six members of the TNF ligand superfamily. The consensus sequences for the CKI motif residues (serine or threonine) are indicated by black shading and proximal acidic residues are indicated in grey shading. In the case of Fas ligand, one out of the three species has a threonine substituted for a serine (indicated below the serine residue). All sequences were obtained from the Swiss protein database and have been predicted from cDNA sequences. The consensus sequence for each member was generated from the number of species indicated in parentheses.

family of cytokines are fascinating, and the scenario, in one case, can be compared with a biological 'handshake', with information passing in both directions as immune cells come into contact. In addition, the role of soluble forms of the TNF receptor superfamily may need to be re-thought, in terms of them acting as physiological ligands.

Materials and methods

Cell line and reagents

The mouse macrophage cell-line RAW264.7 (Ralph and Nakoinz, 1977; Raschke *et al.*, 1978), derived from a BALB/c tumour transformed by Abelson leukaemia virus, was obtained from the American Type Culture Collection (Rockville, MD). Human epithelial kidney 293-T cells were from Edge BioSystems Inc. (Gaithersburg, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). TNFbp, an *E.coli* recombinant-derived, polyethylene glycol-linked, dimeric sTNFR, was provided by Dr Carl Edwards (Amgen, Boulder, CO). BB1101, a hydroxamic acid-based inhibitor of TNF processing, was provided by Dr Andrew Gearing (British Biotech, Oxford, UK). LPS from *E.coli* serotype 055:B5 (Code No. L-2880) was from Sigma. The polyclonal mouse TNF antiserum was developed in rabbit against mature (17 kDa) mouse TNF (Doherty *et al.*, 1992). The specificity of this polyclonal TNF antibody has been confirmed by neutralization of cytotoxicity present on fixed LPS-stimulated RAW264.7 cells towards TNF-sensitive WEHI-164 cells. Also, upon immunoprecipitation of 26 kDa TNF with sTNFR, the antibody will recognize a 26 kDa band by Western blot (unpublished data). TNF1 antibody was a mouse anti-human TNF monoclonal antibody raised against mature human TNF protein (provided by Dr Helmut Holtmann). Polyclonal human type I sTNFR antiserum, 1 mg/ml (Code No. AB-225-PB), was from R&D Systems (Minneapolis, MN). *N*-(2-aminoethyl)-5-chlorisoquinoline-8-sulfonamide, CKI-7 (Chijiwa *et al.*,

1989), was from Toronto Research Chemicals (Ontario, Canada). CKI was a recombinant rat form (Graves *et al.*, 1993) from Calbiochem (La Jolla, CA).

Orthophosphate labelling

Cells were seeded in 6-well plates at 2×10^6 cells/well and allowed to adhere overnight 18–24 h prior to stimulation for 4 h in the presence of LPS (10 μ g/ml) and BB1101 (10 μ g/ml) in phosphate-free DMEM supplemented with phosphate-free FCS (10%). During the last 2.5 h of this stimulation period, the cells were labelled with [32 P]H₃PO₄ (500 μ Ci/ml). Cells treated with sTNFR under various regimes, were subjected to immunoprecipitation for either the membrane-associated 26 kDa TNF (designated mTNF) with anti-sTNFR antibody, or the total cellular 26 kDa TNF from cell lysate with anti-TNF antibody. Membrane-associated TNF was analysed by receptor-mediated ligand precipitation (RMLP) (Crowe *et al.*, 1994). After radiolabelling, the cells were incubated on ice and the unbound sTNFR removed with four washes in phosphate-buffered saline (PBS). The cells were then lysed with an immunoprecipitation lysis buffer [50 mM HEPES buffer pH 7.4, 0.1 M NaCl, 1 mM EGTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride (PMSF)] (de Gunzburg *et al.*, 1989) for 10 min with mild agitation. Immunoprecipitation steps were performed on ice. Lysates were clarified by centrifugation for 10 min at 14 000 g. Mouse TNF antiserum (3 μ l) was added to all samples that were lysed directly after labelling (for the total cellular 26 kDa fraction). To the lysates from the RMLP experiments, 3 μ l of human sTNFR type I antiserum was added. Samples were then incubated for 16 h on a nutator. Immune complexes were collected by incubation with 10 μ l of protein A-Sepharose beads for 3 h on the rotating device. The Sepharose beads were washed twice in radioimmuno-precipitation assay (RIPA) buffer [10 mM Tris-HCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, 500 mM NaCl] and twice in RIPA buffer without NaCl. Each sample was resuspended in 2 $\times$ SDS-PAGE sample buffer, denatured at 100°C for 5 min and separated using 12% SDS-PAGE gels at 140 V. A phosphorimager

(Fujix BAS1000) was used to capture images from the dried gels, and densitometry was performed using Fujix MacBAS v2.0 software.

Mutagenesis of human 26 kDa TNF

The following oligonucleotides were designed for mutagenesis of the cytoplasmic domain of transmembrane TNF from a ($\Delta 1-12$) processing-defective genomic TNF clone (Grell *et al.*, 1995) as follows: wild-type sense, AGGGTACCATGAGCACTGAAAGCATGAT; mutant sense, AGGGTACCATGGCCACTGAAGCCATGATCCGG; antisense, CTT-AGTGGGCGGCCGCACTTCACTGTGC.

Amplification with the antisense and wild-type primers was used to generate the wild-type molecule (with respect to the CKI site). By coincidence, amplification with the mutant primer and the antisense primer resulted in random replacement of the CKI site with a sequence of similar length.

Calcium phosphate-mediated transfection

The 293-T cells were split to a density of 2×10^6 per 9 cm dish. The next day transfection was performed; 10 μ g of plasmid DNA was introduced into a 15 ml snap-cap tube and brought to 455 μ l with H₂O before addition of 45 μ l of 2.5 M CaCl₂. Drop by drop, 500 μ l of $2 \times$ HEPES-buffered saline (HBS) pH 7.1 was added gradually on a vortexer. When gentle precipitates were visible, the DNA was added to the cells. Following incubation for 7 h at 37°C, the culture medium was aspirated and replaced with fresh DMEM. Cells were cultured for a further 18 h post-transfection prior to lysis.

The assay of luciferase activity in transfected 293-T cells was done using d-luciferin (Sigma) as substrate. Cells were lysed by freeze-thaw prior to assay. Light emission was measured using a luminometer, a Biocounter M2500 (Lumac bv, Landgraaf, The Netherlands).

Immunoprecipitation of mTNF and kinase assay using recombinant CKI

Cells were stimulated as above, in DMEM supplemented with 10% FCS, before being washed three times in ice-cold PBS. Following this, PBS was added to some wells, and PBS, NaF (50 mM) and calyculin A (50 nM) to other wells. Cells were incubated for 15 min on ice, then sTNFR (10 μ g/ml) was added. The cells were then incubated for a further 15 min on ice and transferred to 37°C, 5% CO₂ for 15 min. Finally, they were washed three times in ice-cold PBS and lysed in an immunoprecipitation lysis buffer containing NaF (50 mM) and a protease inhibitor cocktail (Boehringer Mannheim, Germany, Cat. No. 1 697 498) for 10 min. Lysates were clarified by centrifugation at 14 400 g for 10 min at 4°C, and incubated overnight at 4°C with 3 μ l of polyclonal anti-sTNFR antibody. Complexes were immunoprecipitated with 30 μ l (50/50 v/v) of protein A-Sepharose (1–3 h on a rotator at 4°C). This was followed by three washes in RIPA buffer with sodium β -glycerophosphate (20 mM), and then one wash in kinase buffer [50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 20 mM sodium β -glycerophosphate, 1 mM PMSF]. The kinase reaction was carried out in 20 μ l of kinase buffer that contained 100 U of CKI and 10 μ Ci of [³²P]ATP per sample. The reaction was incubated at 30°C for 45 min, and terminated by the addition of 30 μ l of $2 \times$ SDS-PAGE sample buffer, and heating to 100°C. Samples were separated by SDS-PAGE and densitometry conducted as described above.

Statistical analysis

Statistical analysis was performed using the software program Instat version 2.03 (GraphPad Software Inc., CA). A Mann-Whitney *U* test was applied for pair-wise comparison of the data.

Intracellular calcium measurement using fluorescence microscopy

Cells cultured on coverslips were incubated in DMEM (without FCS) containing 2 μ M Fura2-AM (Molecular Probes, Oregon) for 20 min at room temperature. Coverslips were rinsed three times with DMEM (without FCS) and incubated in the same medium for a further 20 min at room temperature to allow cleavage of the Fura2-AM ester bond. The coverslips were then washed in physiological salt solution (135 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 5 mM glucose, 1 mM CaCl₂ and 10 mM HEPES, pH 4), fixed onto a chamber containing physiological salt solution and fluorescence was observed with a Fluor 40 $\times$, 1.3 NA oil immersion fluorescence objective, fitted to a Nikon Diaphot inverted microscope. Cells were excited using a Simens Leitz (Germany) 75 W XBO UV lamp, which was coupled to a Lambda 10, Sutter (Novato) filter wheel, containing 340 and 380 nm excitation filters (Omega Optical, Brattleboro). Fura2 fluorescence was observed using a 400DM Nikon

dichroic mirror fitted with a 520–560 BA emission filter to a GEN-II-SYS image intensifier (Dage-MTI, Michigan City). The filter wheel setting and the acquiring and interpretation of emission data were achieved using MCID M2 software (Imaging Research, Ontario, Canada). The software program was calibrated as stated by the manufacturer. Ratio images, 340/380 nm, were taken every 5 min and the [Ca²⁺]_i was calculated using the MCID program. In each case, the initial ratio image was taken as a control before the addition of the sTNFR, which remained in the chamber throughout the experiment. Cells that showed an even distribution of fluorescence and no punctate fluorescence, indicating sequestration by organelles, were selected for measurement.

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