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Regulation of Raf-1 kinase by TNF via its second messenger ceramide and cross-talk with mitogenic signalling

Gertraud Müller[1], Peter Storz, Soizic Bourteele, Heike Döppler, Klaus Pfizenmaier, Harald Mischak[2], Alex Philipp[2], Christian Kaiser[2] and Walter Kolch[3]

Institut für Zellbiologie und Immunologie, Universität Stuttgart, Allmandring 31, D-70569 Stuttgart, [2]GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Klinische Molekularbiologie und Tumorgenetik, München, Germany and [3]Beatson Institute for Cancer Research, Bearsden, Glasgow, Scotland

[1]Corresponding author

Raf-1 kinase is a central regulator of mitogenic signal pathways, whereas its general role in signal transduction of tumour necrosis factor (TNF) is less well defined. We have investigated mechanisms of Raf-1 regulation by TNF and its messenger ceramide in cell-free assays, insect and mammalian cell lines. *In vitro*, ceramide specifically bound to the purified catalytic domain and enhanced association with activated Ras proteins, but did not affect the kinase activity of Raf-1. Cell-permeable ceramides induced a marked increase of Ras–Raf-1 complexes in cells co-expressing Raf-1 and activated Ras. Likewise, a fast elevation of the endogeneous ceramide level, induced by TNF treatment of human Kym-1 rhabdomyosarcoma cells, was followed by stimulation of Ras–Raf-1 association without significant Raf-1 kinase activation. Failure of TNF or ceramide to induce Raf-1 kinase was observed in several TNF-responsive cell lines. Both TNF and exogeneous C_6-ceramide interfered with the mitogenic activation of Raf-1 and ERK by epidermal growth factor and down-regulated v-Src-induced Raf-1 kinase activity. TNF also induced the translocation of Raf-1 from the cytosolic to the particulate fraction, indicating that this negative regulatory cross-talk occurs at the cell membrane. Interference with mitogenic signals at the level of Raf-1 could be an important initial step in TNF's cytostatic action.

Keywords: ceramide/epidermal growth factor/Raf-1 kinase/Ras/tumour necrosis factor

Introduction

Tumour necrosis factor (TNF) is a pleiotropic cytokine capable of inducing, depending on the cell type, strikingly different cellular reactions, such as proliferation, differentiation and necrotic or apoptotic cell death (reviewed in Grell and Scheurich, 1996). Response diversity emanates from two distinct TNF receptors and several receptor-associated signal transducers, comprising the families of death domain proteins and the TNF receptor-associated factor (TRAF) proteins (Rothe *et al.*, 1994; Tewari and Dixit, 1996; Wallach, 1997). TNF-mediated cellular activation also encompasses the rapid activation of phospholipases, such as PC-phospholipase C, phospholipase A_2 and sphingomyelinases, the latter producing the novel second messenger ceramide (Hannun and Obeid, 1995; Obeid and Hannun, 1995). Exogenously added short chain ceramides can mimic several TNF actions (Schütze *et al.*, 1992; Mathias and Kolesnick, 1993; Obeid *et al.*, 1993; Jarvis *et al.*, 1994), emphasizing the role of this lipid messenger in TNF signalling. Ceramide is an important mediator of cell cycle arrest and apoptosis (Hannun and Obeid, 1995; Obeid and Hannun, 1995). Several intracellular targets of ceramide have been described, the role of which in TNF signalling is not yet fully understood. Protein kinase C (PKC) ζ was described as a ceramide-responsive kinase that can function as a molecular switch between pleiotropic signals of TNF (Lozano *et al.*, 1994; Müller *et al.*, 1995) and that very recently has been shown to be an important negative regulator of apoptotic signals induced by UV irradiation (Berra *et al.*, 1997). A ceramide-activated phosphatase has also been described (Dobrowski and Hannun, 1992). Further, TNF induces a ceramide-activated protein kinase (CAPK) located at the plasma membrane, which has been reported directly to phosphorylate and activate Raf-1 *in vitro* and in intact myelomonocytic HL-60 cells (Yao *et al.*, 1995). Whether Raf-1 activation is a general consequence of TNF stimulation, and its specific role in TNF signal pathways are unknown at present.

Raf-1 is considered a key enzyme in transmitting proliferative, developmental and oncogenic signals from receptor tyrosine kinases to the nucleus via the mitogen-activated protein kinases/extracellular signal-regulated protein kinases (MAPKs/ERKs) signal pathway (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). The regulatory signals converging at the Raf kinase are highly complex and still a target of intense research (for review, see Morrison and Cutler, 1997). The activation of Raf-1 in response to many growth factors is associated with increased phosphorylation of the molecule at serine, threonine and, in some cases, tyrosine residues (Heidecker *et al.*, 1992). The mechanism of Raf-1 activation is incompletely understood, but in many situations is dependent on association with activated, i.e. GTP-loaded, Ras (Moodie and Wolfman, 1994; Williams and Roberts, 1994; Dent *et al.*, 1995). Ras-GTP binds to a domain in the amino-terminus of Raf-1 with high affinity (Vojtek *et al.*, 1993; Warne *et al.*, 1993; Chuang *et al.*, 1994). Ras binding in itself fails to activate Raf-1 directly. Recent studies proposed that a physiological role for Ras in Raf-1 activation is to translocate Raf-1 from the cytosol to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), where Raf-1 is activated by a still unknown mechanism involving phosphorylation. Recruitment to

the membrane, however, is not sufficient for maximal activation of Raf-1 kinase (Leevers *et al.*, 1994). In addition to phosphorylation, the involvement of membrane factors and lipids in Raf-1 activation has been suggested (Dent *et al.*, 1995; Ghosh *et al.*, 1996).

The possible participation of lipids in the regulation of Raf-1 activity appears plausible also for structural reasons. Raf-1 contains three domains conserved among members of the Raf kinase family, termed CR1, CR2 and CR3. The CR1 region in the NH_2-terminus harbours sequences involved in Ras–Raf interaction (Chuang *et al.*, 1994; Moodie and Wolfman, 1994; Mott *et al.*, 1996). In addition, CR1 contains two pairs of cysteine residues, which bind zinc and are spaced identically to paired cysteines in members of the PKC family (Quest *et al.*, 1994a,b; Mott *et al.*, 1996). This zinc finger motif is doubled in most members of the PKC family and is required for binding of diacylglycerol (DAG) or phorbol esters (Hug and Sarre, 1993; Goodnight *et al.*, 1994; Dekker *et al.*, 1995). The atypical PKC ζ possesses only one zinc finger motif, which shares homology with the Raf-1 zinc finger region. Neither the zinc finger of Raf-1 nor that of PKC ζ interact with DAG or phorbol esters (Goodnight *et al.*, 1992; Kazanietz *et al.*, 1994). PKC ζ, however, binds ceramide and is activated by this lipid in a biphasic manner (Lozano *et al.*, 1994; Müller *et al.*, 1995).

In this study, we show that Raf-1 binds ceramide, and we have examined whether ceramide as a TNF-induced lipid messenger plays a direct role in modulating Raf kinase interaction with its upstream regulators and/or downstream effector molecules. Moreover, we investigated a potential cross-talk of TNF with mitogenic signalling cascades and provide evidence for a ceramide-mediated negative regulation at the level of Raf-1 kinase.

Results

Raf-1 kinase specifically binds [^{14}C]ceramide in the kinase domain, but is not activated by ceramide

TNF-induced intracellular elevations of ceramide are expected to result in direct interaction of this lipid messenger with effector proteins. Therefore, we tested whether ceramide might be able to interact with Raf-1 kinase. For this purpose, a GST-tagged Raf-1 kinase was expressed in the baculovirus/Sf-9 cell system and affinity purified by adsorption to glutathione–Sepharose (GS) beads. GST–Raf-1-loaded GS beads were incubated with [^{14}C]ceramide as described in Materials and methods. As shown in Figure 1A, purified GST–Raf-1 specifically bound [^{14}C]ceramide in a saturable manner. To correct for unspecific binding of ceramide to the GS beads and/or the GST portion of the fusion protein, parallel experiments were performed with GS beads loaded with the GST protein alone. Under these conditions, no binding of [^{14}C]ceramide above background levels was obtained (data not shown). This indicates that ceramide specifically binds to the Raf-1 kinase.

To test whether ceramide affects the catalytic activity of Raf-1, the kinase activity of purified GST–Raf-1 was examined with kinase-negative MEK (MEK^-) as substrate (inset in Figure 1A). Addition of increasing amounts of C_{16}-ceramide to the *in vitro* kinase assays did not change the basal phosphotransferase activity of Raf-1. Consistent

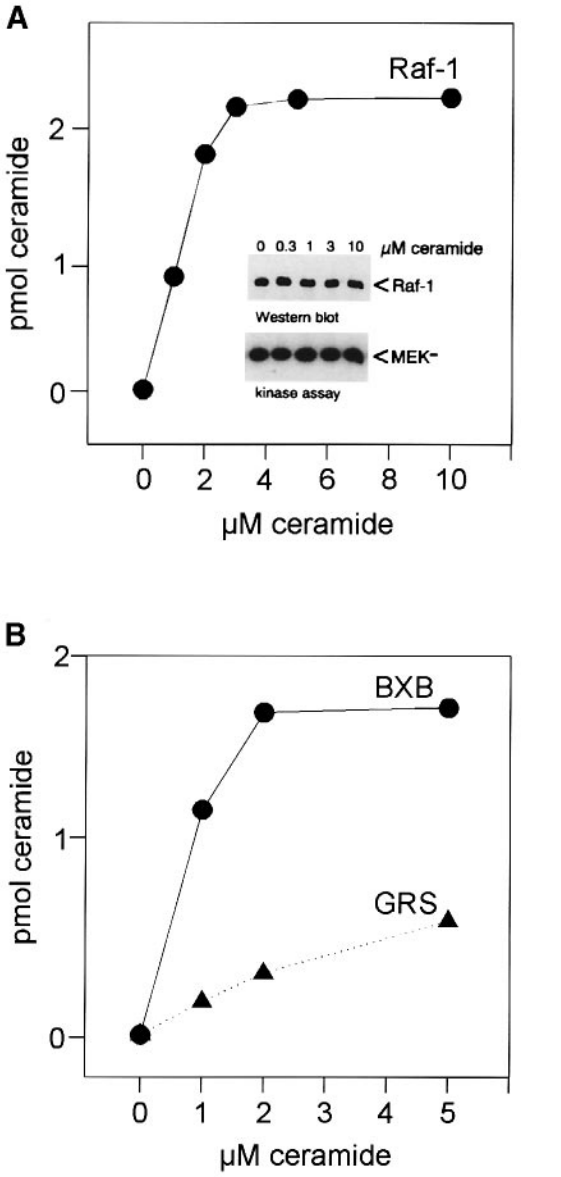

Fig. 1. Saturation binding of [^{14}C]ceramide to Raf-1 kinase. (**A**) GST–Raf-1 proteins (~ 200 ng/vial) immobilized on glutathione–Sepharose beads were incubated with [^{14}C]ceramide for 2 h in the presence or absence of 100-fold excess of unlabelled C_{16}-ceramide at 4°C and extensively washed, and radioactivity was determined as described in Materials and methods. Shown is specific binding (unspecific binding subtracted from total binding) as mean values from triplicate determinations. Similar data were obtained in three independent experiments. Insert: the kinase activity of purified GST–Raf-1 was measured in the presence of increasing amounts of C_{16}-ceramide, using kinase-negative MEK (MEK^-) as substrate. Samples were separated on a 10% SDS gel and transferred to nitrocellulose. The blot was autoradiographed ('kinase assay'), and the Raf-1 proteins present in the reactions subsequently were visualized by staining with a Raf-1-specific antiserum ('Western blot'). (**B**) [^{14}C]ceramide binds to the Raf-1 kinase domain. The Raf-1 kinase domain (BXB, ●) and the Raf-1 regulatory domain Raf-1 (GRS, ▲) were purified as recombinant GST fusion proteins from Sf-9 cells. Binding of [^{14}C]ceramide was assayed as described in Materials and methods and in (A).

results were obtained with synthetic C_2- and C_6-ceramides, or when activated Raf-1 was used. Activated Raf-1 was isolated from Sf-9 cells co-infected with GST–Raf-1 and Ras plus Lck or PKC α, respectively. The co-expression

led to substantial activation of Raf-1 (depending on the preparation; between 15- and 25-fold in the case of Ras plus Lck, and between 4- and 6-fold in the case of PKC α), but again ceramide did not alter Raf-1 activity significantly (data not shown). These results indicate that ceramide binding does not modulate Raf-1 kinase activity directly in a cell-free assay system.

To determine the potential interaction site of ceramide, GST fusion proteins encompassing only the regulatory (GRS) or the catalytic domains (BXB) of Raf-1 kinase were tested for binding of [^{14}C]ceramide (Figure 1B). Contrary to our expectation that ceramide would bind to CR1 in the regulatory domain of the Raf-1 kinase, we found specific saturable binding curves only with the catalytic domain of the Raf-1 kinase in a range of 1–5 μM of ceramide. The BXB-binding curve closely matched those obtained with full-length Raf-1, whereas no saturable binding was observed with the GRS fragment, suggesting that ceramide specifically associated with the Raf-1 kinase domain.

Ceramide enhances the association of the Raf-1 kinase with Ras-GTP in vitro

Since no change in the activity of Raf-1 kinase towards its downstream substrate, MEK, was obtained in the presence of ceramide, the interaction with its upstream activator, Ras, was investigated (Figure 2A). GST–Raf-1 was immobilized on GS beads and pre-incubated with increasing concentrations of C_{16}-ceramide. Then, excess amounts of either Ras-GTP or Ras-GDP were added and, after washing, bound Ras proteins were detected by immunoblotting. Ceramide did not induce binding of Raf-1 to Ras-GDP, but selectively enhanced the association of Raf-1 with Ras-GTP. At ceramide concentrations >0.5 μM, the Ras-GTP binding to the Raf-1 kinase increased in a dose-dependent manner, reaching saturation at ~2 μM (Figure 2B). This binding profile closely matched the ceramide-binding curve to Raf-1, suggesting that ceramide induces a conformational change in Raf-1, which elevates its affinity towards Ras-GTP. Ceramide-mediated enhancement of Ras-GTP binding to Raf-1 requires the biologically active form of ceramide and is specific for this messenger, as neither the dihydro form of C_6-ceramide nor several other lipid mediators were found to stimulate Ras–Raf-1 interaction (Figure 2C).

Ceramide stimulates Ras association with Raf-1 in Sf-9 cells

The *in vitro* findings pertaining to the enhancement of Ras interaction with Raf-1 by ceramide could be reproduced *in vivo*, using Sf-9 cells co-expressing GST–Raf-1 and v-Ha-Ras. Stimulation of Sf-9 cells with cell-permeable ceramides increased the association of Ras-GTP with the Raf-1 kinase, peaking at 10 min of ceramide stimulation (Figure 3). In a parallel kinase assay, where kinase-negative MEK$^-$ was added as substrate, no significant increase in either the phosphorylation of MEK$^-$ or Raf-1 autophosphorylation could be detected. Similarly, ceramide treatment did not enhance the kinase activity of Raf-1 isolated from Sf-9 cells co-expressing wild-type (wt) Ha-Ras, v-Ha-Ras plus Lck, wt Ha-Ras plus Lck or PKC α, respectively (data not shown). These findings are fully consistent with the *in vitro* experiments, indicating

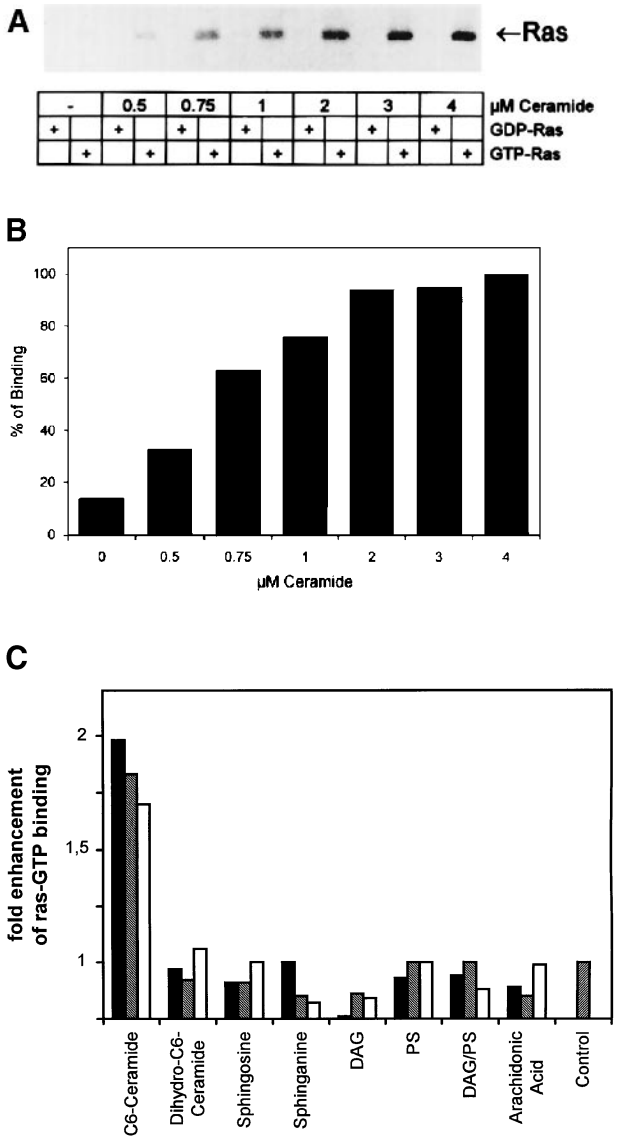

Fig. 2. Ceramide specifically enhances binding of Ras-GTP to Raf-1 *in vitro*. (**A**) GST–Raf-1 was incubated with equal amounts of purified Ras proteins loaded with GTP or GDP in the presence of increasing concentrations of C_{16}-ceramide. The binding reactions were washed, separated by SDS–PAGE and blotted. Associated Ras was stained with the LA69 anti-Ras monoclonal antibody. (**B**) Densitometric quantitation of the Western blot shown in (A). (**C**) Lipid specificity of enhancement of the Ras–Raf-1 interaction. The assay was performed as in (A) with three concentrations [black, 5; shaded, 1; white, 0.5 μM (μg/ml in the case of PS)] of the lipid mediators as indicated or no lipid added to Raf-1 during the pre-incubation (negative control). Data are expressed as fold enhancement of Ras binding, with background binding to untreated Raf-1 kinase set at 1.0.

that ceramide binding is not sufficient to stimulate the Raf-1 kinase activity in this cellular system.

Ceramide does not stimulate Raf-1-mediated phosphorylation and activation of MEK-1

To investigate the potential impact of ceramide on downstream signalling events, Sf-9 cells were infected with a virus expressing GST-tagged MEK-1 alone or in combination with a Raf-1 virus (Figure 4). Cells were stimulated with cell-permeable ceramide for 5–60 min. GST–MEK-1 was isolated and its kinase activity was monitored using

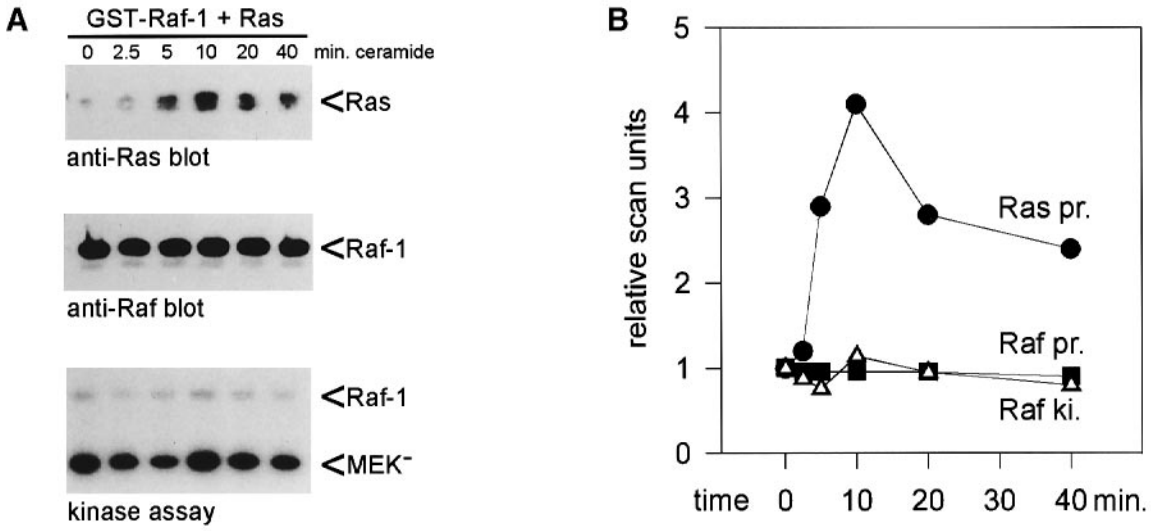

Fig. 3. Ceramide stimulates Ras association with Raf-1 *in vivo* and in Sf-9 cells. (**A**) GST–Raf-1 was co-expressed with Ha-Ras in Sf-9 cells. Three days post-infection, cells were treated with membrane-permeable ceramides (20 μM C_2- and C_6-ceramide). At the time points indicated, cells were lysed. GST–Raf-1 was purified by adsorption to glutathione–Sepharose beads and examined for kinase activity with kinase-negative MEK (MEK^-) as substrate ('kinase assay'). Samples were separated by SDS–PAGE, blotted to nitrocellulose and sequentially stained with Ras- (upper panel) or Raf-1- (middle panel) specific antibody. (**B**) Quantitation of the blots shown in (A). ●, Ras protein associated with GST–Raf-1; ■, GST–Raf-1 protein; △, Raf-1 kinase activity.

kinase-negative MAP kinase ($MAPK^-$) as substrate. Activation of MEK was strictly dependent on the co-expression of Raf-1, but ceramide stimulation of the cells had no influence on MEK-1 activity or on its interaction with Raf-1. Again, similar results were obtained when GST–MEK-1 was co-expressed with Raf-1 plus v-Ras. Despite the fact that v-Ras co-expression caused a further increase in MEK-1 activation, ceramide treatment had no effect on induced MEK-1 activity (data not shown). Taken together, these results demonstrate that neither Raf-1 nor its immediate downstream target, MEK-1, is activated by ceramide, despite the increase in Raf-1–Ras interaction mediated by ceramide.

TNF treatment enhances the association of Raf-1 with activated Ras in mammalian cells, but has no impact on basal or Ras-stimulated Raf-1 kinase activity

The data obtained *in vitro* and in Sf-9 cells argued against a direct role for ceramide in Raf-1 activation. However, an indirect role could not be excluded. For example, ceramide could affect unknown signalling molecules absent in Sf-9 cells or in the *in vitro* assays performed with purified recombinant proteins. Moreover, synthetic ceramide analogues might not be able to substitute fully for endogenous ceramides generated in the mammalian cell. TNF is a well-studied physiological activator of ceramide generation. Therefore, we also investigated whether TNF could activate the Raf-1 kinase in mammalian TNF-responsive cells. In the first set of experiments, we used Kym-1 cells, which are highly sensitive to TNF and undergo apoptosis upon TNF stimulation within 18 h (Grell *et al.*, 1993). In these cells, TNF induced the production of intracellular ceramide within 2 min; elevated levels of ceramide were sustained for at least 30 min (Figure 5A, and data not shown). Subsequent to the elevation of intracellular ceramide concentrations, there was a >200% increase in association of Ras with Raf-1

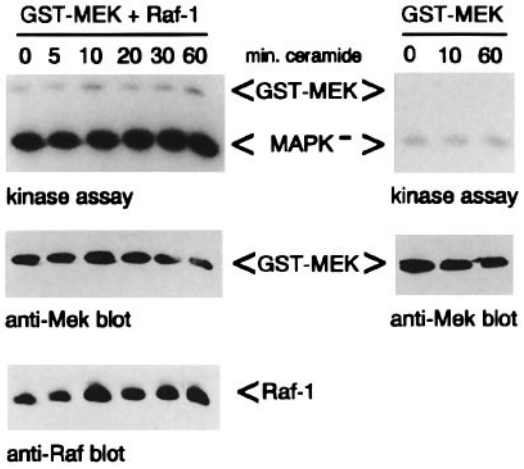

Fig. 4. Ceramide does not affect phosphorylation and activation of MEK by Raf-1 kinase. GST–MEK was expressed in Sf-9 cells alone (right panel) or together with Raf-1 (left panel). GST–MEK was isolated from C_6-ceramide-treated cells by adsorption to glutathione–Sepharose beads and subjected to an *in vitro* kinase assay with kinase-negative MAPK ($MAPK^-$) as substrate. Reactions were separated by SDS–PAGE and blotted. Blots were autoradiographed (upper panel) and stained for MEK (middle panel) and associated Raf-1 (lower panel) with anti-MEK and anti-Raf-1 antisera, respectively.

kinase, as shown by co-immunoprecipitation experiments. However, TNF failed to cause a significant stimulation of the Raf-1 kinase activity in these cells (Figure 5B).

In the next set of experiments, a detailed analysis of TNF effects was conducted in NIH-3T3 fibroblasts not sensitive to TNF-induced apoptosis. A pool of NIH-3T3 transfectants, termed NIH/Raf-1, stably overexpressing a human Raf-1 cDNA ~6-fold over endogenous levels, were used. Overexpression of Raf-1 does not activate the MEK/MAPK cascade (data not shown), but these cells were chosen to facilitate the detection of weak stimulatory responses. Serum-starved cells were treated with 100 ng/ml murine TNF for the times indicated. The kinase activity

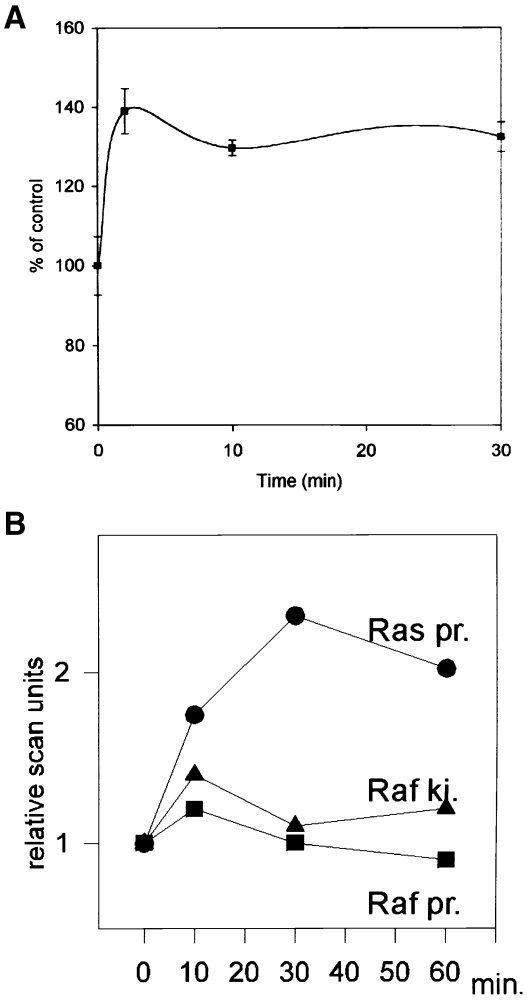

Fig. 5. TNF increases the intracellular ceramide level and stimulates Ras association with Raf-1 in Kym-1 cells. (**A**) Kym-1 cells (5×10^6) were stimulated by TNF (10 ng/ml). At the indicated times, cells were washed and scraped from the plates for lipid extraction. Ceramide levels were determined using the DAG kinase assay kit (Amersham) with ceramide as standard. Three independent experiments were performed; values are the means of triplicate determinations. The basal intracellular ceramide level (100%) corresponds to 24.6 pmol of ceramide-1-phosphate per 5×10^5 cells. (**B**) Kym-1 cells were stimulated with TNF (10 ng/ml) and lysed at the time points indicated. Anti-Raf-1 immunoprecipitates from 5×10^6 cells were separated by SDS–PAGE, blotted to nitrocellulose and subsequently stained with Ras- or Raf-1-specific antibodies. In parallel, the Raf-1 immunoprecipitates were examined for kinase activity in a linked kinase assay using MEK and kinase-negative MAPK ($MAPK^-$) as substrates. The amounts of Raf-1 and co-immunoprecipitated Ras proteins were quantified by laser densitometry. Raf-1 kinase activity was measured using a Fuji PhosphoImager. ●, co-precipitated Ras; ▲, Raf-1 kinase activity; ■, Raf-1 protein.

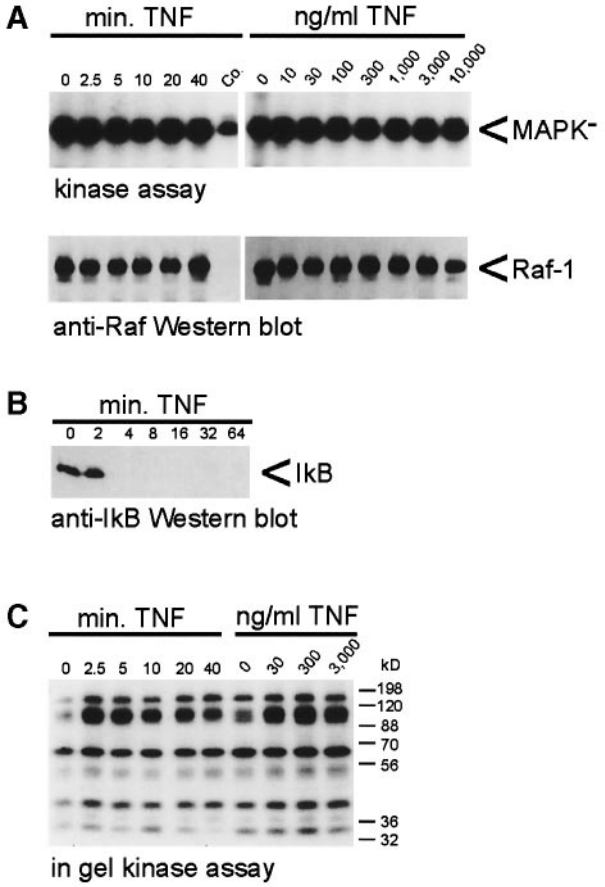

Fig. 6. TNF induces IκB proteolysis and activation of 50 and 97 kDa kinases but fails to activate the Raf-1 kinase. (**A**) NIH/Raf-1 cells were treated with 100 ng/ml murine TNF for the times indicated or with different amounts of TNF for 10 min, respectively. The kinase activity of Raf-1 immunoprecipitates was determined in a linked kinase assay. In the lane 'Co.', MEK and $MAPK^-$ were incubated in the absence of a Raf-1 immunoprecipitate. (**B**) In parallel, the same lysates were immunoblotted and stained for IκB. (**C**) Aliquots of the lysates used in (A) were analysed by an in-gel kinase assay with MBP as substrate polymerized into the gel. Shown is the autoradiogram of the dried gel. The sizes of molecular weight markers are indicated.

of Raf-1 immunoprecipitates was measured in linked kinase assays with MEK and kinase-negative MAPK as substrates, revealing no activation of Raf-1. Even the use of a wide range of different concentrations of TNF (10–10 000 ng/ml) failed to activate the Raf-1 kinase (Figure 6A). The lack of Raf-1 stimulation was not due to a general absence of TNF responses in these cells. TNF or ceramide rapidly triggered IκB proteolysis (Figure 6B) and stimulated several myelin basic protein (MBP) kinases detectable by in-gel kinase assays (Figure 6C). Notably, TNF readily enhanced the actvity of 34, 50 and 95/97 kDa MBP kinases. As judged from the apparent molecular weight, the 50 kDa band probably corresponds to SAPKs/JNKs, while the 95/97 kDa band may represent CAPK. Thus, TNF was able to elicit specific responses in these cells, including the activation of kinases, which, however, were different from Raf-1. Completely consistent results were obtained when NIH-3T3 or NIH/Raf-1 cells were treated with cell-permeable ceramides (C_2- and C_6-ceramide). These ceramides induced IκB degradation and MBP kinases, but did not activate the Raf-1 kinase within a concentration range of 0.1–300 μg/ml (data not shown).

As Raf-1 activation by TNF may be cell type-specific (Yao *et al.*, 1995; Verheij *et al.*, 1996), in addition to NIH-3T3 and Kym-1 cells, we also examined other known TNF-responsive cell lines, in particular Jurkat, HL-60, COS, HUVEC (data not shown) and HeLa cells (see below). However, under the conditions described above, neither TNF nor ceramide caused a significant activation of Raf-1 in any of these cells lines including HL-60, where Raf-1 activation by CAPK has been reported previously (Yao *et al.*, 1995).

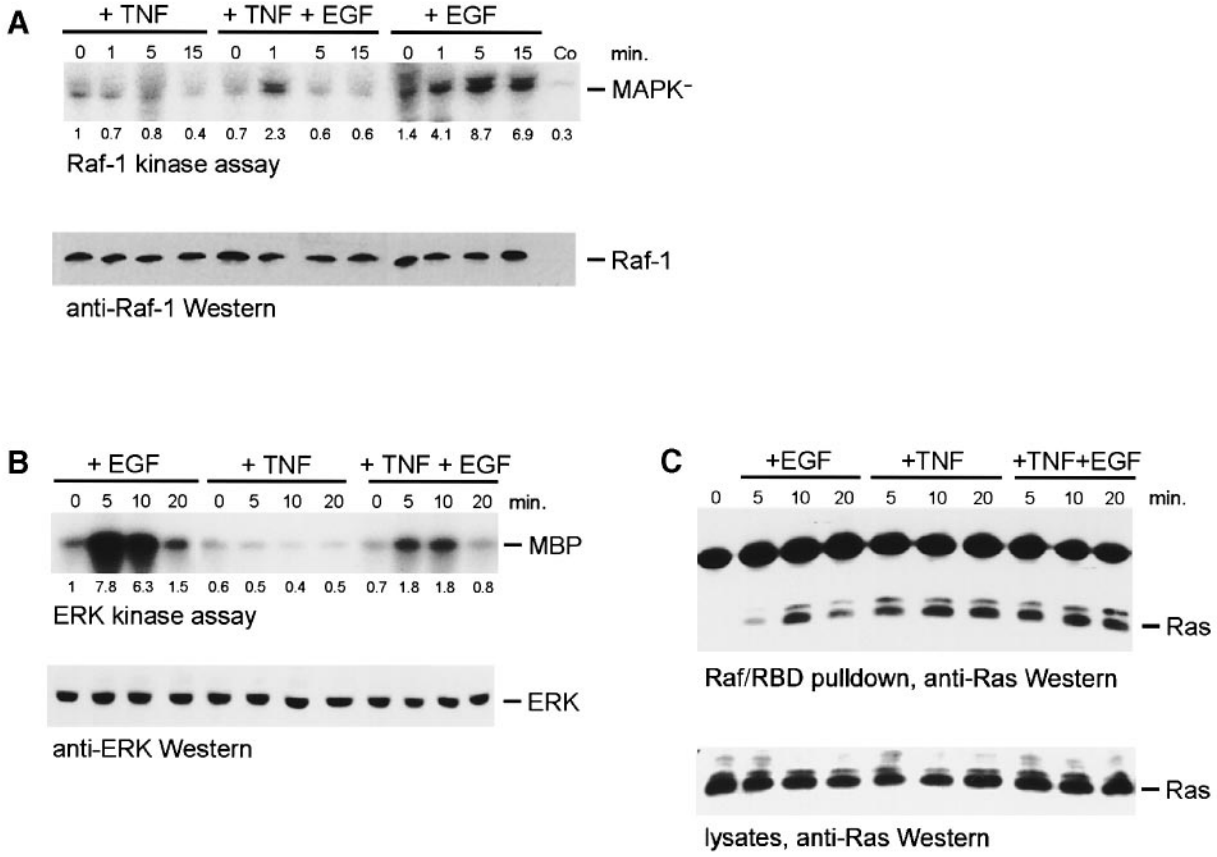

Fig. 7. TNF interferes with EGF-induced activation of the mitogenic signal cascade downstream of Ras at the level of Raf-1. Serum-starved HeLa cells were used throughout the experiments described below, treated with 10 ng/ml TNF, 25 ng/ml EGF or both for the time points indicated and analysed for *in vivo* activation of Ras, Raf-1 and ERK. TNF was administered immediately before EGF. (**A**) Inhibition of EGF-induced Raf-1 kinase activity by TNF. Cells were lysed, Raf-1 was immunoprecipitated with antibody cRafVI and subjected to a linked kinase assay with Rec, MEK-1 and kinase-inactive ERK-2 ($MAPK^-$) as substrates. The reactions were separated by 10% SDS–PAGE and blotted. The blot was autoradiographed (Raf-1 kinase assay) and subsequently stained with Raf-1-specific antiserum (anti-Raf-1 Western). Kinase activities were quantified with a Fuji PhosphoImager and are given as relative scan units (untreated cells = 1) below the lanes. In the lane 'Co', MEK-1 and $MAPK^-$ were incubated in the absence of a Raf-1 immunoprecipitate as a negative control. (**B**) Inhibition of EGF-induced ERK kinase activity by TNF. ERK-1 and -2 were immunoprecipitated with a mixture of anti-ERK-1 and -2 agarose beads (5 µl each, Santa Cruz Biotechnology) and tested for kinase activity with MBP as substrate. The reactions were resolved on 12.5% SDS–PAGE and autoradiographed (ERK kinase assay). Substrate phosphorylation was quantified as in (A) and is indicated as relative units below the lanes. In parallel, an aliquot of the same cell lysates was blotted and stained with an anti-pan ERK antibody (Transduction Labs) to ensure equal ERK expression (ERK Western blot). (**C**) Activation of Ras by TNF and EGF. The activation of endogenous Ras was measured by pulldown assays with a GST fusion protein comprising the Ras-binding domain of Raf-1 (Raf/RBD) as described in Materials and methods. Samples were separated on a 12.5% SDS gel and blotted for associated Ras with a panRas antibody (Transduction Labs). The total content of cellular Ras in the same lysates was determined by direct Western blotting.

TNF and C_6-ceramide down-regulate the activation of the Raf-1 kinase by EGF

The observation that TNF or ceramide stimulated the formation of Raf-1–Ras complexes without significantly activating the Raf-1 kinase suggested that TNF may fail to provide an accessory signal necessary to activate Raf-1 when bound to Ras. Alternatively, it is conceivable that ceramide binding to Raf-1 imposes, despite enhanced Ras–Raf interaction, a negative signal for subsequent activating events. Therefore, we investigated whether TNF might modulate Raf-1 activation by stimuli, such as epidermal growth factor (EGF), which efficiently activate Ras as well as Raf-1 (reviewed in Downward, 1996; Marrais *et al*., 1996). For this purpose, HeLa cells were treated with TNF, EGF or a combination of both agents, and the cellular activation of Ras, Raf-1 and ERK was studied (Figure 7). While EGF readily stimulated both Raf-1 and ERK kinase activity, evident from $MAPK^-$ and MBP substrate phosphorylation, TNF did not increase either Raf-1 or ERK kinase activity (Figure 7A and B). However, TNF efficiently counteracted the EGF-mediated activation of both enzymes, dampening both the amplitude and the duration of activation (Figure 7A and B). TNF was clearly capable of activating Ras *in vivo*, with a more rapid and sustained activation compared with the transient response to EGF stimulation, as revealed from pulldown of activated GTP-Ras by a GST–Raf–Ras-binding domain (RBD) fusion protein (Figure 7C). The kinetics and levels of GTP-Ras upon combined EGF/TNF treatment of cells closely resembled that of cells treated with TNF only (Figure 7C).

With respect to down-modulation of EGF-induced Raf-1 kinase activity by TNF, exogenous C_6-ceramide mimicked TNF action (Figure 8A). Together with the data shown in Figure 7, these results suggest that TNF, via its second messenger ceramide, interferes with EGF-induced mitogenic signal cascades downstream of Ras at the level of Raf-1 activation, probably at the cell membrane. In support

of this reasoning, a TNF-dependent translocation of Raf-1 to cellular membranes without kinase activation was noted (see below). The above experiment performed with HeLa cells has been repeated with the monocytic cell line HL-60, in which the MAPK cascade was strongly activated by phorbol ester (TPA) treatment of cells instead of EGF. As for HeLa cells (Figure 7), simultaneous treatment with TNF (10 ng/ml) or C_6-ceramide down-regulated TPA-induced Raf-1 and ERK activation in HL-60 cells as well. Moreover, TNF and C_2-ceramide reduced TPA- and EGF-induced ERK stimulation in NIH-3T3 cells and v-Src-induced ERK stimulation in COS cells (data not shown).

It has been reported that kinases can provide the second signal required for full Raf-1 activation at the cell membrane (Marais *et al.*, 1995). Therefore, the effects of TNF were examined in this setting. For this purpose, COS-1 cells were transfected with Raf-1 alone or together with v-Ras, v-Src or a combination of both, and treated with TNF (Figure 8B and C). TNF induced a translocation of Raf-1 to cellular membranes (Figure 8B), but again failed to enhance the basal (Figure 8B and C) and v-Ras-stimulated (Figure 8C, upper panels) Raf-1 kinase activity in these cells. In contrast, TNF induced a rapid and sustained suppression of Raf-1 activation by v-Src in the absence and presence of v-Ras (Figure 8C, lower panels). These data are consistent with the hypothesis that TNF inhibits activation of Raf-1 by secondary signals at the cell membrane.

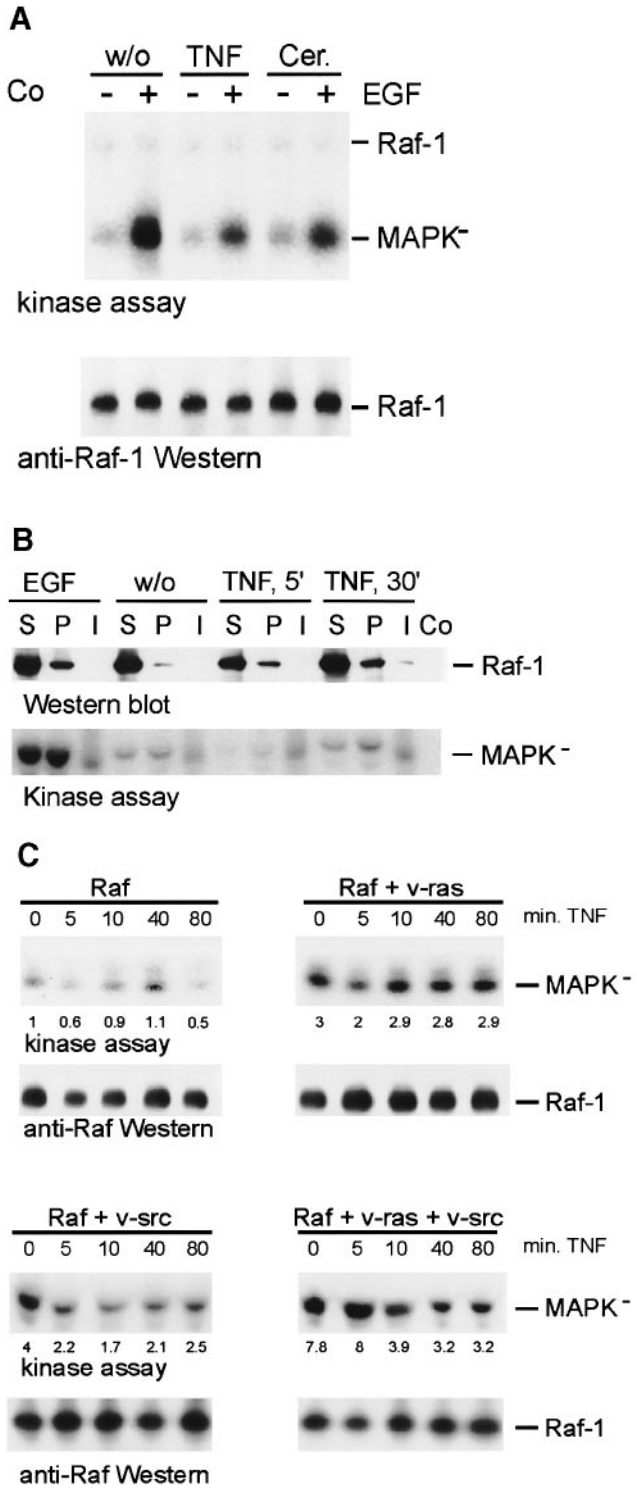

Fig. 8. (**A**) Inhibition of EGF-induced Raf-1 kinase activity by ceramide. COS cells were transfected with Raf-1. Two days post-transfection, cells were serum deprived for 16 h and subsequently treated for 10 min as indicated. Human TNF (10 ng/ml) or C_6-ceramide (50 μM) was administered immediately before EGF (25 ng/ml). Raf-1 immunoprecipitates were examined for kinase activity using the linked kinase assay with MEK and kinase-negative MAPK ($MAPK^-$) as substrates. (**B**) TNF induces redistribution of Raf-1 to cellular membranes without activating Raf-1. COS cells were transfected with Raf-1. Serum-starved cells were treated with EGF for 10 min or TNF for 5 or 30 min, respectively. Cells were fractionated into soluble cytoplasmic ('S'), membrane ('P') and insoluble ('I') components. The distribution of Raf-1 was examined by Western blotting, and the kinase activity was monitored by the linked kinase assay, which tests for MEK activation. (**C**) TNF interferes with v-Src-induced Raf-1 activation. COS-1 cells were transiently transfected with the indicated plasmids. Serum-starved cells were treated with 10 ng/ml TNF. The kinase activity of Raf-1 immunoprecipiates was measured in a linked kinase assay.

Discussion

It is well accepted that Raf-1 takes a central position in mitogenic signal pathways induced by a variety of growth factors (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). However, the involvement and functional role of Raf-1 in signal pathways of the pro-inflammatory cytokine TNF is less well understood, and controversial data concerning TNF-dependent activation of Raf-1 exist. Thus it was reported recently by several groups that TNF treatment can stimulate Raf-1 kinase activity (Yao *et al.*, 1995; Huwiler *et al.*, 1996; Kalb *et al.*, 1996; Modur *et al.*, 1996). Yao *et al.* (1995) have proposed that CAPK can directly phosphorylate and activate Raf-1 *in vitro* and in HL-60 cells, resulting in the subsequent activation of MEK. In contrast, other studies conducted in different cell lines did not find TNF activation of Raf-1 (Westwick *et al.*, 1994; Winston *et al.*, 1995; Gardner and Johnson, 1996), suggesting that TNF-mediated stimulation of Raf-1 may be highly cell type-specific. In the present investigation, we employed cell-free *in vitro* assays and studied various TNF-responsive cell lines for their linkage of Raf-1 to TNF signal pathways. Our data provide compelling evidence that Raf-1 is modulated by both TNF and its immediate intracellular messenger ceramide. We show that TNF treatment via ceramide enhanced Ras-GTP binding to Raf-1, yet, contrary to the expectations from

data of some of the previous reports (Yao *et al.*, 1995; Huwiler *et al.*, 1996; Kalb *et al.*, 1996; Modur *et al.*, 1996), TNF did not activate Raf-1 kinase. Rather, we provide evidence for a negative regulatory cross-talk of TNF, via its second messenger ceramide, with typical mitogenic signals at the level of Raf-1. As TNF's down-regulating function of mitogen-induced Raf-1 kinase activity was paralleled by the ceramide-dependent binding of Ras-GTP and recruitment of Raf-1 to cell membranes, a causal relationship is suggested. We propose that ceramide imposes a dominant-negative effect on mitogenic Raf-1 activation by sequestration into inactive Ras–Raf-1 complexes. The alkyl chain protrusion model of ceramide function recently proposed by Krönke (1997), according to which ceramide primarily serves as membrane lipid anchor to recruit proteins to membranes rather than as a soluble messenger, is in line with our interpretation.

Interestingly, ceramide specifically bound to the catalytic domain of Raf-1. Binding of this lipid messenger was expected to occur at the regulatory domain of Raf-1 in structural analogy to another cysteine-rich zinc finger motif found in PKC ζ (Hug and Sarre, 1993; Goodnight *et al.*, 1994; Dekker *et al.*, 1995), the latter being activated by ceramide upon TNF stimulation (Lozano *et al.*, 1994; Müller *et al.*, 1995). The affinity of ceramide binding to Raf-1 was found to be rather low, with an estimated K_D of 1 μM, and did not result in activation of the kinase. For comparison, PKC ζ contains both low- and high-affinity ceramide-binding sites; ceramide binding to the low-affinity site of PKC ζ did not result in activation, whereas high-affinity binding caused robust activation of the enzyme (Müller *et al.*, 1995). By analogy, since Raf-1 lacks a high-affinity ceramide-binding site, ceramide may not be expected to activate Raf-1.

Recently, two phospholipid-binding domains in Raf-1 were described. Phosphatidylserine (PS) binds to the cysteine-rich amino-terminal region (Huwiler *et al.*, 1996), while the second messenger phosphatidic acid (PA) binds to the carboxy-terminal domain (Ghosh *et al.*, 1996). PS did not affect either Ras-GTP binding or the activity of the Raf-1 kinase *in vitro* (Force *et al.*, 1994; Figure 2C), whereas PA was suggested to promote the translocation of Raf-1 kinase to the cell membrane (Williams and Roberts, 1994). Although the latter study failed to show binding of type IV ceramide from bovine brain to Raf-1, this result is not necessarily in contradiction to the data presented here and probably reflects the use of different ceramides: C_{16}-ceramide used here is the prevalent physiological ceramide in non-brain tissue, whereas bovine brain ceramides are a mixture of predominantly longer chain ceramides.

The important finding of the work presented here is that binding of ceramide to Raf-1 is functionally relevant, as it directly enhanced the association with GTP-loaded Ras without kinase activation. The membrane translocation and interference with mitogenic activation of Raf-1 kinase by both TNF and exogeneous ceramide is thus probably a direct consequence of ceramide binding to Raf-1. Interestingly, the ceramide-binding domain is located in the C-terminal kinase domain of Raf-1, whereas the RBD is situated in the amino-terminus. Nevertheless, the ceramide-mediated increase in Raf-1–Ras association is specific: it can be saturated, requires a GTP- and not a GDP-loaded Ras and its binding curve closely parallels the ceramide-binding profile. Moreover, neither two other lipid messengers also implicated in TNF signal pathways, DAG and arachidonic acid, nor metabolic derivatives of ceramide were found to stimulate Ras–Raf-1 interaction, corroborating that this action is ceramide-specific. The ceramide-induced increase of Ras-GTP binding is only observed with full-length Raf-1, not with the isolated Raf-1 kinase domain (BXB), indicating that ceramide does not shift the binding epitope for Ras-GTP to BXB. Therefore, it may be concluded that ceramide modulates the affinity of Raf-1 for Ras-GTP, probably by inducing a conformational change. A conformational switch in Raf-1 between states with low and high affinity for Ras-GTP is consistent with the observation that the isolated RBD has a substantially higher affinity for Ras-GTP than full-length Raf-1 (Chuang *et al.*, 1994). Based on the data presented, we propose that the ceramide-induced changes in Ras–Raf interaction itself and/or other conformational alterations due to ceramide binding to the Raf-1 catalytic domain interfere with the inducibility of the kinase by mitogenic signals.

With respect to TNF's role in Raf-1 kinase activation, the controversial findings so far published can be explained in several ways. First, TNF may activate the Raf-1 kinase towards a yet unknown substrate different from MEK. This possibility can be considered because the CAPK-induced phosphorylation of Raf-1 was mapped to threonines 268/269 (Yao *et al.*, 1995), which represent the major Raf-1 autophosphorylation sites *in vitro* (Morrison *et al.*, 1993). These sites, however, differ from known activating phosphorylation sites, but might serve to redirect the substrate specificity of Raf-1. Second, TNF may fail to recruit a cofactor required for activation of the Raf-1 kinase after binding to Ras-GTP. The availability of this cofactor might be cell-specific. In support of this hypothesis, recent work has shown that association of Raf-1 with Ras-GTP may occur without a concomitant activation of Raf-1 (Moodie *et al.*, 1994). Our data obtained with several cell lines treated only with TNF would be compatible with such a model. However, since both TNF and exogeneous ceramide each interfered with a simultaneous EGF-induced or v-Scr-mediated Raf-1 and ERK activation, we favour a third alternative, namely an active down-regulating function of TNF on Raf-1 kinase activation. Although the molecular mechanisms of this negative regulatory cross-talk are not yet elucidated in detail, our results indicate that binding of the TNF messenger ceramide to the catalytic domain of Raf-1 is a critical event. The rapid *in vivo* generation of ceramide upon TNF stimulation and its accumulation in cells where TNF exerts cytostatic or cytotoxic action (Hannun, 1996; G.Müller, S.Bourteele, A.Haußer, H.Döppler, G.Schwarzmann and K. Ffizenmaier, in preparation) are consistent with the sustained and dominant inhibition of mitogenic signals fuelling the MAPK cascade observed here.

As TNF/ceramide sequesters active Ras-GTP into an inactive Ras–Raf complex, with direct consequences for the mitogenic activation of ERK, we also propose that this sequestration may limit the availability of functional Ras for other downstream components such as GTPase-activating protein, phosphatidylinositol-3′-kinase (PI-3 kinase) and Ral-GDP dissociation stimulator (Ral-GDS).

The latter two molecules are intimately involved in mitogenic signalling. Ral-GDS regulates the Ral GTPase, which participates in the activation of phospholipase D (reviewed in Feig *et al.*, 1996), while PI-3 kinase mediates proliferative responses as well as protection from apoptosis (reviewed in Vanhaesebroeck, 1996). Raf-1 also appears to be part of at least two different anti-apoptotic signalling pathways. One is the ERK pathway, which, in addition to the transduction of mitogenic signal(s), has been suggested to provide protection against apoptosis (Xia *et al.*, 1995; Cuvillier *et al.*, 1996; Gardner and Johnson, 1996). Another is the bcl-2 pathway, which recently has been reported to use Raf-1 as an effector kinase that may disable the death-promoting properties of BAD (a member of the bcl-2 gene family) by direct phosphorylation (Wang *et al.*, 1996). Oncogenic Raf-1 alleles reduce apoptosis induced by growth factor withdrawal (Cleveland *et al.*, 1994; Wang *et al.*, 1996), the latter often leading to very high and persistent intracellular ceramide levels (reviewed in Hannun, 1996). Therefore, it is conceivable that the formation of inactive Raf-1–Ras complexes, mediated by ceramide, at the same time counteracts both mitogenic and anti-apoptotic signals. This may represent a novel regulatory principle of ceramide, which could be important for one or more of the pleiotropic actions of TNF, in particular growth arrest, cellular differentiation and apoptosis.

Materials and methods

Reagents and cells

Semisynthetic C_2-, C_6- and C_{16}-ceramides, sphingosine and sphinganine were purchased from Biomol, bovine brain type IV and III ceramides, DAG, arachidonic acid and PS were obtained from Sigma, [^{14}C]palmitoylsphingosine (C_{16}-ceramide), [γ-^{32}P]ATP, [^{32}P]$_i$ and the DAG kinase assay were from Amersham, human TNF (2×10^7 U/mg) was a kind gift of BASF and Günter Eißner, GSF, murine TNF was purchased from Boehringer Mannheim. NIH-3T3, NIH/Raf, COS-1 and HeLa cells were maintained in Dulbecco's modified minimal essential medium (DMEM, Gibco/BRL) supplemented with 10% bovine calf serum (BCS, Seromed). Jurkat and HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Seromed). Kym-1 cells were cultured in Click's RPMI with 10% FCS. NIH/Raf cells are a mass culture of NIH-3T3 cells that had been infected with a recombinant retrovirus transducing a human Raf-1 cDNA along with a hygromycin B resistance gene (pLXSH/Raf, generously provided by John Sedivy). After selection with hygromycin B, >200 resistant cell clones were pooled and designated NIH/Raf. These cells overexpress the human Raf-1 ~6-fold above the endogenous Raf-1. Consistent with previous reports (Heidecker *et al.*, 1990), Raf-1 overexpression did not lead to cell transformation. For transient transfections, COS-1 cells were seeded into 6-well plates in 2 ml of DMEM containing 10% NU serum (Collaborative Research, #55000). To 20–40% confluent cells, 20 µl of DC mix and 3 µg of plasmid DNA were added; 100 ml of DC mix contains 1.03 g of chloroquin phosphate (Sigma) and 8 g of DEAE–dextran (Sigma, D-9885). Three to four hours later, the medium was replaced by phosphate-buffered saline (PBS) containing 10% dimethylsulfoxide (DMSO) for 2 min. Then, cells were fed with DMEM plus 10% FCS. Under these conditions, protein expression increased linearly between 1 and 10 µg of plasmid DNA transfected. Two days post-transfection, cells were deprived of serum overnight and treated as described in the figure legends.

Expression and purification of GST-tagged proteins

The construction of a baculovirus transducing a GST-tagged Raf-1 has been described previously (Häfner *et al.*, 1994). Briefly, the human c-Raf-1 cDNA was cloned in-frame downstream of the GST portion of the baculovirus transfer vector pGSTAcC5. The pGSTAcC5 vector (a kind gift from Gisela Heidecker) features a GST portion followed by thrombin cleavage site, a five glycine linker region and a polylinker. In BXB, most of the Raf-1 regulatory domain was eliminated by the deletion of amino acids 26–303 (Bruder *et al.*, 1992). GRS represents a Raf-1 mutant, where an internal *Stu*I fragment encoding amino acids 332–597 was deleted. This results in removal of almost the entire kinase domain CR3. To construct GST–MEK-1, two novel restriction sites were introduced into the MEK-1 cDNA (Gardner *et al.*, 1993), an *Nco*I site at the start codon and an *Xba*I site at the stop codon. Then, the 1.2 kb *Nco*I–*Xba*I fragment was cloned into the pGSTAcC5 vector cut with *Nco*I and *Xba*I. Sf-9 cells were infected with recombinant baculoviruses and harvested on day 3 post-infection. Cells were lysed in 2 ml of TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) plus protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin] and phosphatase inhibitors (100 µM orthovanadate, 20 mM β-glycerophosphate, 2 mM sodium fluoride and 2 mM sodium pyrophosphate). The lysate was centrifuged for 10 min at 15 000 r.p.m. and the supernatant was incubated with 100 µl of GS (Pharmacia) for 15 min at 4°C under constant agitation. The beads were pelleted (1 min, 15 000 r.p.m., 4°C) and washed twice with 500 µl of TBST and four times with binding buffer [20 mM Tris–HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.05% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF and 1 µg/ml leupeptin]. These protein preparations were used for *in vitro* binding assays and kinase assays. The GST–Raf/RBD expression vector was constructed by inserting a 0.57 kb *Nco*I–*Hin*dIII fragment of the human Raf-1 cDNA comprising amino acids 1–150 into *Escherichia coli* expression vector pGEX-KG. The recombinant protein was purified by adsorption to GS beads (Pharmacia) according to standard protocols and used for detection of activated Ras protein in cell lysates (see below).

Raf-1 and MEK-1 kinase assays and Western blotting

Cells were lysed as described above. GST-tagged Raf-1 or MEK proteins were isolated from Sf-9 cell lysates by affinity adsorption to GS (Pharmacia). In order to obtain activated GST–Raf-1, Sf-9 cells were co-infected with GST–Raf-1 and Ras plus Lck or PKC α, respectively, as described previously (Häfner *et al.*, 1994). From lysates of mammalian cells, Raf-1 was immunoprecipitated with 1.5 µl of c-RafVII antiserum (Häfner *et al.*, 1994) plus 10 µl of protein-A–agarose (Boehringer). Precipitates were washed three times in TBST and once with Raf kinase buffer (20 mM Tris–HCl, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). Raf kinase reactions contained 100 ng of purified recombinant kinase-negative MEK (MEK$^-$), or, in the case of linked kinase assays, 50 ng of kinase-competent MEK plus 150 ng of kinase-negative MAPK (MAPK$^-$), 2 µM ATP and 5 µCi of [γ-^{32}P]ATP (Häfner *et al.*, 1994). MEK activity was assayed under the same conditions using 150 ng of MAPK$^-$ as substrate. After incubation for 30 min at 25°C, reactions were terminated by boiling in SDS gel sample buffer, separated by SDS gel electrophoresis and transferred to nitrocellulose membranes. The blots were autoradiographed and subsequently stained with the indicated antibodies using enhanced chemiluminescence (ECL kit, Amersham) for detection. Substrate phosphorylation was quantitated with a Fuji PhosphoImager. In-gel kinase assays were performed exactly as described by Krautwald *et al.* (1995).

Ras activation assay

This assay is based on the fact that only activated, i.e. GTP-loaded, Ras is capable of binding to the Raf-1 RBD. Thus, Raf/RBD-associated Ras proteins reflect the fraction of activated Ras molecules. The assay was performed essentially as described by Rooij and Bos (1997). Briefly, cells pre-treated with various stimuli as indicated in the figure legends were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS). Lysates were cleared by centrifugation at 15 000 *g* for 5 min at 4°C. The supernatant was incubated with 5 µg of the GST-tagged Raf/RBD bound to GS beads for 20 min. Samples were washed four times with 1 ml of RIPA buffer, separated on 12.5% SDS–PAGE and blotted. Associated Ras proteins were visualized with a Ha-Ras antibody (Transduction Laboratories).

[^{14}C]ceramide-binding assays

For the [^{14}C]ceramide-binding assays, GST–Raf-1 was purified from Sf-9 cells as described above. Aliquots of 150 ng of GST–Raf-1 immobilized on GS beads were washed three times for 10 s with 1 ml of binding buffer (20 mM Tris–HCl pH 7.4; 10 mM $MgCl_2$; 50 mM NaCl) and resuspended in 1 ml of buffer. To this suspension, different concentrations of labelled [^{14}C]ceramide with and without a 100-fold excess of unlabelled *N*-hexadecanoyl ceramide (C_{16}-ceramide), for determination of total and unspecific binding, respectively, were added. Prior to the assay, the stock solutions of unlabelled ceramide (100×) in kinase buffer were treated for 10 min in an ultrasonic water bath at 37°C.

Incubation was performed for 2 h at 4°C and unbound [^{14}C]ceramide was removed by washing the GST–Raf-1 beads three times for 10 s in binding buffer. Unspecific binding was dependent on the amount of Sepharose beads present but, under the conditions employed here, did not exceed 10% of total binding. The amount of [^{14}C]ceramide present in the precipitates was measured by liquid scintillation counting.

Ras–Raf binding assay

Aliquots of 300 ng of GST–Raf-1 immobilized to GS beads were added to 500 µl of binding buffer (20 mM Tris–HCl pH 7.4; 10 mM $MgCl_2$; 50 mM NaCl; 0.05 % Triton X-100) containing increasing concentrations of C_{16}-ceramide or other lipid mediators and incubated for 2 h at room temperature. Excess lipids were removed by washing once with binding buffer. To reduce unspecific binding, 2 µl of ELISA blocking reagent (1 mg/ml, Boehringer Mannhein) was added. After 10 min incubation at 4°C, the GST–Raf-1 beads were washed once and resuspended in binding buffer. Purified Ha-Ras protein (a kind gift from Alan Wolfman) was loaded with a non-hydrolysable GDP analogue (beta-thio) or with a non-hydrolysable GTP analogue (GMP-PNP) according to the method of DiBattiste *et al*. (1993). For specific binding, 1 µg of Ha-Ras-GTP or Ha-Ras-GDP was added to the GST–Raf and incubated for 1 h at 4°C. Unbound Ras proteins were removed by repeated washes with binding buffer before the samples were separated by SDS–PAGE and blotted onto nitrocellulose. Ha-Ras associated with Raf-1 was detected by immunoblotting with the monoclonal anti-Ras antibody LA69 (Quality Biotech).

Co-precipitation of Ras with Raf-1

Kym-1 cells were stimulated with 10 ng/ml TNF and, at the indicated times, plates were placed on ice, medium was removed and cells were washed twice with ice-cold PBS. Cells were scraped from the plates with a rubber policeman in buffer A (Müller *et al.*, 1995) and the immunoprecipitation with Raf antiserum was performed according to the procedure described in Müller *et al.* (1995). SDS–PAGE and Western blot analysis of co-precipitated Ras were done as described above. The combined Raf-1 kinase assay containing MEK^+ and $MAPK^-$ was performed as described above. Intracellular ceramide levels were determined with the DAG kinase assay kit (Amersham).

Cell fractionation

Cells were serum starved overnight and treated as described in the figure legends. After washing with ice-cold PBS, cells were lysed in isotonic buffer (20 mM Tris–HCl pH 7.4, 0.3 M sucrose, 1 mM PMSF, 1 µg/ml leupeptin, 100 µM orthovandate, 20 mM β-glycerophosphate, 2 mM sodium pyrophosphate) by mild sonication. Nuclei were removed by centrifugation at 3000 *g* for 10 min. The supernatant was centrifuged further at 100 000 *g* for 60 min. The supernatant corresponding to the soluble cytosolic fraction ('S') was adjusted to 1% Triton X-100 and used for immunoprecipitation. The pellet ('P') was washed three times by resuspension in isotonic buffer, and subsequently extracted in an equal volume of this buffer containing 1% Triton X-100 for 1 h on ice to recover detergent-soluble Raf-1. Insoluble matter ('I') was removed by centrifugation for 10 min at 10 000 *g*. The insoluble fraction did not contain kinase activity towards MEK, when assayed as described by Stokoe *et al.* (1994). The Triton X-100-soluble fraction was immunoprecipitated with Raf-1 antiserum. The quality of each fractionation experiment was assured by examining the distribution of Ras, PKC α and PKC ε between the S100 and P100 fractions by immunoblotting. Ras was only found in the P100 fraction. PKC α was detected solely in the S100 fraction in serum-starved cells, but completely redistributed to the P100 fraction after TPA stimulation. PKC ε was found in both the S100 and P100 fractions in serum-starved cells, but accumulated markedly in the P100 fraction after TPA treatment (not shown).

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