Mitogenic signalling pathway of tumour necrosis factor involves the rapid tyrosine phosphorylation of 41 000- M_r and 43 000- M_r cytosol proteins

Michiaki KOHNO,*† Naomi NISHIZAWA,* Masafumi TSUJIMOTO† and Hiroshi NOMOTO*

*Department of Biology, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502, and †Suntory Institute for Biomedical Research, Mishima, Osaka 618, Japan

Tumour necrosis factor (TNF) is a potent mitogen for some fibroblast cell lines. Here we have examined the TNF-mediated changes in protein phosphorylation in Swiss 3T3 and human FS-4 fibroblasts, and compared them with changes observed after the treatment of cells with other mitogens, such as platelet-derived growth factor (PDGF) and bombesin. TNF stimulated the rapid phosphorylation of two 41 000- M_r and two 43 000- M_r cytosol proteins on tyrosine, threonine and/or serine, as did PDGF, epidermal growth factor and fibroblast growth factor; the increased levels of this mitogen-induced protein-tyrosine phosphorylation correlated well with the extent of mitogen-induced DNA synthesis as determined by the percentage of labelled nuclei. In contrast, bombesin, which is an even better mitogen for Swiss 3T3 cells than TNF, stimulated the tyrosine phosphorylation of 41 000- M_r and 43 000- M_r proteins only to a limited extent. On the other hand, bombesin and PDGF stimulated the rapid serine phosphorylation of an 80 000- M_r acidic protein, a major substrate for protein kinase C; increased phosphorylation of the 80 000- M_r protein was not observed at all when cells were stimulated with TNF. These results suggest significant differences among the mitogenic signalling pathways of TNF, PDGF and bombesin as regards the involvement of protein kinases; the mitogenic signalling pathway of TNF involves the activation of tyrosine kinase, but not of protein kinase C, whereas bombesin seems to transduce its mitogenic signal mainly through the activation of protein kinase C, and the activation of both kinases seems to be involved in the mitogenic signalling pathway of PDGF.

INTRODUCTION

Tumour necrosis factor (TNF) is a protein originally identified as a mediator of antitumour activity in animals injected sequentially with Calmette-Guérin bacillus and endotoxin [1]. TNF has cytostatic or cytotoxic activity on certain tumour cells, which is mediated by its binding to specific receptors. A variety of malignant as well as normal cells of various tissues express high-affinity receptors for TNF [2–6]; the presence of TNF receptors on many cell types is not surprising, in view of the wide variety of biological responses that TNF elicits in different cells. For example, TNF stimulates the secretion of collagen and prostaglandin E_2 in synovial cells and dermal fibroblasts [7], induces the expression of major histocompatibility complex Class I antigens in vascular endothelial cells and fibroblasts [8], stimulates osteoblast-mediated bone resorption [9], activates neutrophils [10], induces interleukin-1 production in endothelial [11] and mononuclear cells [12], and enhances the response of activated T-cells [13]. Other studies also have indicated that TNF stimulates the growth of fibroblasts [14]. These pleiotropic activities suggest that the main physiological role of TNF is as a mediator of inflammation [15]. The mechanisms by which TNF induces such diversity in cellular responses are still unclear. Although sensitivity to TNF can be modulated by down-regulation of TNF-binding capacity [16], TNF responsiveness itself is not related to the number or affinity of TNF-binding sites, suggesting important post-receptor mechanisms controlling the tissue-specific cellular TNF responses. To date, however, little is known about the molecular basis of these TNF-mediated signalling mechanisms.

In many peptide-hormone receptor systems, signal

transduction to the nucleus involves the activation of protein kinases (for review, see ref. [17]). Receptor molecules for EGF, PDGF and insulin have inherent hormone-dependent tyrosine kinase activity (for review, see ref. [18]): rapid tyrosine phosphorylation of 41 000- M_r and 43 000- M_r proteins has been commonly observed after the activation of quiescent cells with a variety of growth factors [19–21], although the kinase which directly phosphorylates those proteins is still unknown. A group of peptide hormones such as PDGF, bombesin and thrombin have been shown to stimulate phosphoinositide breakdown, leading to the generation of diacylglycerol, which activates protein kinase C (for review, see refs. [22] and [23]); the 80 000- M_r acidic cellular protein is a major substrate of protein kinase C [24–26]. Recently, the participation of protein kinases in TNF signal transduction has also been suggested, since protein kinase inhibitors can block the TNF-mediated induction of its own gene [27].

In order to investigate further whether protein kinases are involved in the mitogenic signal transduction of TNF, we have studied protein-phosphorylation patterns in TNF-sensitive fibroblasts. In this report, we show that the activation of tyrosine kinase(s), but not of protein kinase C, is involved in the mitogenic signalling pathway of TNF.

MATERIALS AND METHODS

Cell culture

Human skin fibroblasts (used between passages 10 and 15) were provided by Dr. S. Toyama (Kyoto University, Kyoto, Japan); human foreskin fibroblasts (FS-4) were from Dr. J.

Abbreviations used: TNF, tumour necrosis factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BrdU, 5-bromo-2'-deoxyuridine.

† To whom correspondence and reprint requests should be addressed.

Vilček (New York University Medical Center, New York, NY, U.S.A.); Swiss albino mouse fibroblasts (Swiss 3T3; American Type Culture Collection, CCL92) were obtained through the Japanese Cancer Research Resources Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum for human fibroblasts, or 10% (v/v) calf serum for Swiss 3T3 cells. Human fibroblasts were arrested in the G₀/G₁-phase by incubating in DMEM containing 2% fetal-calf serum for 3 days, whereas Swiss 3T3 cells were growth-arrested by incubation in serum-free medium [DMEM containing 2 mg of BSA/ml (Boehringer, Mannheim, Germany), 10 µg of soybean lipid/ml (Boehringer), 20 nM-progesterone, 30 nM-Na₂SeO₃ and 10 mM-Hepes, pH 7.4] for 36 h; then growth factors were added.

Radiolabelling of cells and cell lysis

For labelling with [³²P]P_i, cells were growth-arrested identically as described above, except that the concentration of phosphate in each medium was lowered to 0.1 mM. Labelling was accomplished by adding [³²P]P_i (carrier-free; ICN Biomedicals, Costa Mesa, CA, U.S.A.) at a concentration of 1 mCi/ml to the cells for 16 h. Growth factors were then added and the cells were incubated for 10 min. Labelled cultures were chilled, washed twice with

ice-cold phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-Na₂HPO₄, pH 7.4), then lysed and treated with nucleases as described previously [21,22].

Measurement of growth stimulation

Rates of DNA synthesis were measured 20 h after exposure of the cells to mitogens by addition to the cultures of [*methyl*-³H]thymidine (Amersham Japan, Tokyo, Japan) or of 5-bromo-2'-deoxyuridine (BrdU) followed by incubation for 4 h. After incorporation, acid-insoluble radioactivity was determined as described previously [20], or the percentage of BrdU-labelled nuclei was determined with a Cell Proliferation Kit (Amersham Japan). The percentage of labelled nuclei was determined for a total of more than 500 nuclei. Essentially the same results were obtained by these two methods.

Two-dimensional gel electrophoresis

Cell lysates were subjected to isoelectric focusing in 9.2 M-urea for 7000 V·h with pH 5–8 ampholytes, followed by SDS/polyacrylamide-gel electrophoresis on 10%-acrylamide slab gels [21,22]. For the detection of alkali-resistant phosphodiester bonds in phosphoproteins, gels were incubated, after fixation, in 1 M-NaOH at 45 °C for 1.5 h, then neutralized

Table 1. Effect of mitogen treatment on DNA synthesis and alkali-resistant phosphorylation of pp41-A and pp43-A in Swiss 3T3 and FS-4 cells

Cultures of quiescent Swiss 3T3 and FS-4 cells were exposed to mitogens, and BrdU-labelled nuclei were determined in duplicate cultures as described in the Materials and methods section. The relative cell response to each mitogen is indicated as a percentage in proportion to PDGF-stimulated DNA synthesis (in Swiss 3T3 cells) and basic-FGF (b-FGF)-stimulated DNA synthesis (in FS-4 cells). The experiments below were performed on sister cultures corresponding to those shown in the following Figures: Expt. 1 and Figs. 1 and 5; Expt. 4 and Fig. 2, where cells were labelled with [³²P]P_i, then treated for 10 min with mitogens. Samples of the cell lysates corresponding to 4 × 10⁴ Swiss 3T3 cells or 2 × 10⁴ FS-4 cells were analysed by two-dimensional gel electrophoresis, and the gels were treated with 1 M-NaOH. Relative intensity of pp41-A and pp43-A spots in each mitogen-stimulated batch of cells was quantified by scanning the autoradiographs on an LKB laser densitometer; it is indicated as a percentage in proportion to PDGF-stimulated phosphorylation (in Swiss 3T3 cells) and b-FGF-stimulated phosphorylation (in FS-4 cells). Experiments with Swiss 3T3 cells were repeated four times, and experiments with FS-4 cells three times. Results were always essentially the same, and some of them are shown. Abbreviation used: ND, not determined.

Expt. no.	Cells	Treatment	Mitogen-induced DNA synthesis		Phosphorylation of (relative intensity):	
			BrdU-labelled nuclei (%)	Relative response	pp41-A	pp43-A
1.	Swiss 3T3	Control	0.3	(0)	0	0
		TNF (20 ng/ml)	7.4	(23.3)	30.0	28.1
		Bombesin (20 nM)	9.3	(29.5)	13.5	11.8
		PDGF (10 ng/ml)	30.8	(100)	100	100
		TPA (100 ng/ml)	7.7	(24.0)	ND	ND
2.	Swiss 3T3	Control	1.0	(0)	0	0
		TNF	8.8	(20.5)	18.1	20.8
		Bombesin	9.1	(21.2)	5.7	7.1
		PDGF	39.1	(100)	100	100
3.	Swiss 3T3	Control	0.5	(0)	0	0
		TNF	6.9	(12.3)	14.2	17.0
		Bombesin	12.5	(24.0)	8.3	7.5
		PDGF	52.5	(100)	100	100
4.	FS-4	Control	0.2	(0)	0	0
		TNF (20 ng/ml)	7.1	(27.7)	27.8	32.5
		EGF (50 ng/ml)	20.8	(82.7)	91.7	107
		b-FGF (5 ng/ml)	25.1	(100)	100	100
5.	FS-4	Control	0.3	(0.3)	0	0
		TNF	9.0	(25.1)	25.1	29.9
		EGF	36.3	(104)	98.5	108.5
		b-FGF	34.9	(100)	100	100

and dried as described previously [21]. ^{32}P -labelled polypeptides were located by autoradiography, by using a fluorescent screen. For the measurement of pH profile, a pI calibration kit (BDH, Poole, Dorset, U.K.) was used; marker proteins were detected by Coomassie Blue staining after the first-dimension isoelectrofocusing tubular-gel run in parallel. M_r markers were located on slab gels by staining. The gels are shown with the basic end at the left.

Quantitative determination of stimulation of phosphorylation

Samples of the cell lysates containing equal amount of protein were subjected to two-dimensional gel electrophoresis. The relative intensity of the spots of interest was quantified by scanning the resulting autoradiographs on an LKB laser densitometer.

Phosphoamino acid analysis

Portions from non-treated gels were incubated in 5.7 M-HCl for 1 h at 110 °C, and released phosphoamino acids were separated by one-dimensional thin-layer electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1:10:189, by vol.) as described previously [20].

Peptide mapping by limited proteolysis

One-dimensional peptide mapping was carried out as described

by Cleveland *et al.* [28], by using *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, IN, U.S.A.).

Subcellular fractionation

Subcellular fractionation into soluble and particulate fractions was carried out by the procedure described by Cooper & Hunter [29] with some modifications. ^{32}P -labelled cultures were washed twice with cold phosphate-buffered saline, and 20 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-NaCl, 1 mM-MgCl₂, 1% Aprotinin (Sigma Chemical Co.), 1 mM-ATP, 1 mM-phenylmethanesulphonyl fluoride and 0.1 mM-sodium orthovanadate was added. The cells were allowed to swell for 10 min, and then were collected with a rubber policeman and homogenized by pipetting the suspension 20 times with a disposable 26-gauge needle syringe on ice. The homogenate was centrifuged in a SW50.1 rotor at 35000 rev./min for 60 min to separate the particulate fraction of the pellet from the soluble fraction of the supernatant. Both fractions were adjusted to 9.5 M-urea, 4% Nonidet P40, 5% 2-mercaptoethanol and 2% ampholyte, pH 5–8; then equal portions of each sample were analysed by two-dimensional gel electrophoresis.

Materials

Recombinant human TNF was produced in *Escherichia coli* and purified to homogeneity as described previously [30]. EGF

Fig. 1. Alkali-resistant phosphoproteins of Swiss 3T3 cells

Growth-arrested Swiss 3T3 cells were labelled with [^{32}P]P_i, then treated for 10 min with no addition (a), 20 ng of TNF/ml (b), 20 nM-bombesin (c) and 10 ng of PDGF/ml (d). Labelled phosphoproteins were analysed by two-dimensional gel electrophoresis, and the gels were treated with 1 M-NaOH as described in the Materials and methods section. Each gel contained the lysates of about 4×10^4 cells. Arrowheads indicate the positions of two 43000- M_r and two 41000- M_r proteins. Similar results were obtained in four independent experiments. Mobilities of M_r markers [phosphorylase b (94000), BSA (67000) and ovalbumin (43000)] and mobilities of pI markers [metmyoglobin (horse) (7.3), metmyoglobin (pig) (6.45), trifluoroacetylated metmyoglobin (pig) (5.92) and azurin (*Pseudomonas aeruginosa*) (5.65)] are indicated in (d).

Fig. 2. Alkali-resistant phosphoproteins of FS-4 cells

Growth-arrested FS-4 cells were labelled with [32 P]P_i, then treated for 10 min with no addition (a), 5 ng of basic FGF/ml (b), 50 ng of EGF/ml (c) and 20 ng of TNF/ml (d). Alkali-resistant phosphoproteins were analysed as described in the legend of Fig. 1. Each gel contained the cell lysates of about 2×10^4 cells. Arrowheads indicate the positions of two 43000- M_r and two 41000- M_r proteins. Similar results were obtained in three independent experiments.

purified from mouse submaxillary glands and basic FGF purified from bovine pituitary glands were purchased from Toyobo Co. (Osaka, Japan). Recombinant human PDGF (*c-sis*) was from Amersham Japan. Bombesin and TPA were from Sigma Chemical Co.

RESULTS

TNF stimulates tyrosine phosphorylation of 41000- and 43000- M_r cytosol proteins in quiescent fibroblasts

The addition of recombinant human TNF to G₀/G₁-phase-arrested cultures of mouse and human fibroblasts stimulated DNA synthesis (Table 1); mitogenic activity of TNF was dose-dependent, and maximal at concentrations of 15–20 ng/ml. TNF was a rather weak mitogen for the cells, and its effect was always less than those of PDGF, EGF and basic FGF. Bombesin and TPA were also mitogenic for Swiss 3T3 cells and stimulated DNA synthesis to approximately the same level as did TNF.

Under such conditions, Swiss 3T3 and FS-4 cells, labelled for 16 h with [32 P]P_i, were exposed to mitogens for 10 min, lysed, and the proteins were analysed by two-dimensional gel electrophoresis. Before autoradiography, the gels were treated with alkali to hydrolyse most of the serine-bound phosphate groups [31]. PDGF, EGF and basic FGF markedly increased alkali-resistant phosphorylation of two 41000- M_r and two 43000- M_r proteins in mouse and human fibroblasts (Figs. 1 and 2); the apparent isoelectric points of the two 43000- M_r phosphoproteins

in mouse cells (6.69 and 6.57) were slightly more acidic than those in human cells (6.75 and 6.62), whereas those of the 41000- M_r phosphoproteins were identical in human and mouse cells (7.00 and 6.86).

When 32 P-labelled and PDGF-stimulated human fibroblasts were homogenized and fractionated in the presence of Mg²⁺, two 41000- M_r and two 43000- M_r phosphoproteins were present in the high-speed supernatant fraction (Fig. 3b). PDGF also induced the alkali-resistant phosphorylation of a 36000- M_r protein in human fibroblasts [20], which was present in the particulate fraction under the cell fractionation conditions described above (Fig. 3c). When cells were fractionated in the presence of 1 mM-EDTA but not of Mg²⁺ ions, the 36000- M_r phosphoprotein was localized in both particulate (~60%) and soluble (~40%) fractions; the distribution of the 41000- M_r and 43000- M_r phosphoproteins in the soluble fraction was not affected by the presence of EDTA (results not shown). The 36000- M_r protein, which was first identified as a major substrate for the tyrosine kinase encoded by the oncogene from Rous sarcoma virus [32], and thereafter referred to as Calpactin I, is an abundant protein and is located in the lamina region underlying the plasma membrane; it binds to membrane and phospholipid vesicles, as well as actin and spectrin, in a Ca²⁺-dependent fashion [33,34]. Thus the bivalent-cation-dependent distribution of the 36000- M_r phosphoprotein in the particulate fraction was as expected, and was consistent with previously described results [29,33]. Two 41000- M_r and two 43000- M_r phosphoproteins were present in

Fig. 3. Localization of two 43000- M_r and two 41000- M_r phosphoproteins and one of 36000 M_r .

Growth-arrested human skin fibroblasts were labelled with [³²P]P_i, then treated with 10 ng of PDGF/ml for 10 min. One control culture (not shown; phosphoprotein spots seen in PDGF-treated cultures were undetectable, as shown in Figs. 1a and 2a) and one PDGF-treated culture (a) were prepared directly for two-dimensional gel electrophoresis. One PDGF-treated culture was washed, and the cells were homogenized and separated into soluble (b) and particulate (c) fractions as described in the Materials and methods section. Equal portions of each sample (equivalent to the lysates of about 1×10^4 cells) were analysed. All gels were incubated in 1 M-NaOH before being exposed. Arrowheads indicate positions of phosphoproteins: two of 43000 M_r , two 41000 M_r and one 36000- M_r .

the soluble fraction under both cell-fractionation conditions, suggesting that all these proteins were localized in the cytosol fraction.

TNF also stimulated the alkali-resistant phosphorylation of two 41000- M_r and two 43000- M_r proteins in mouse and human cells. Phosphorylation appeared rapidly and was maximal 5 min after the exposure of cells to TNF, as was the case for other mitogens such as PDGF and EGF described previously (results not shown; [20]). The phosphoproteins from TNF-treated Swiss 3T3 cells were excised from the non-alkali-treated gels and subjected to partial acid hydrolysis. As shown in Fig. 4, all four phosphoproteins contained phosphotyrosine. In addition, more acidic forms of 41000- M_r (pp41-A) and 43000- M_r (pp43-A) phosphoproteins contained phosphoserine and phosphothreonine, whereas their more basic forms (pp41-B, pp43-B) contained phosphoserine. Peptide-map analysis by partial proteolysis with *Staph. aureus* V8 protease of ³²P-labelled 41000- M_r and 43000- M_r proteins from Swiss 3T3 cells treated with TNF and PDGF showed identical proteolytic cleavage patterns for each phosphoprotein, regardless of the mitogen used, indicating that TNF and PDGF induced phosphorylation at the same site(s) on each protein (results not shown; [20]).

The increased level of alkali-resistant phosphorylation of 41000- M_r and 43000- M_r proteins induced by TNF was lower compared with those by PDGF, EGF and basic FGF, and was parallel with the lower mitogenic activity of TNF. Changes in the

extent of mitogen-stimulated phosphorylation of the major forms of 41000- M_r and 43000- M_r proteins, pp41-A and pp43-A, were quantified by laser densitometry of the autoradiographs. As shown in Figs. 1 and 2 and Table 1, the increased levels of TNF-, PDGF-, EGF- and basic-FGF-induced phosphorylation of 41000- M_r and 43000- M_r proteins correlated well with the extent of these mitogen-induced DNA syntheses as determined by the percentage of labelled nuclei. Increased phosphorylation of those proteins was not observed in TNF-treated L929 cells, for which TNF was highly cytotoxic (results not shown). All these results suggested that tyrosine phosphorylation of such a common set of cytosol proteins may play an important role in the mitogenic signalling pathways of TNF, PDGF, EGF and basic FGF. In contrast, bombesin, which was an even better mitogen for Swiss 3T3 cells than was TNF, stimulated the alkali-resistant phosphorylation of 41000- M_r and 43000- M_r proteins, but only to a limited extent (Fig. 1c and Table 1). Bradykinin was also a good mitogen for Swiss 3T3 cells, but it did not stimulate the tyrosine phosphorylation of those proteins significantly (M. Kohno, unpublished work).

TNF does not stimulate the phosphorylation of 80000- M_r protein

Mitogens such as PDGF, bombesin and thrombin have been shown to stimulate the rapid turnover of phosphoinositides, leading to increases in intracellular concentrations of both Ca²⁺

Fig. 4. Phosphoamino acid analysis of two 43000- M_r and two 41000- M_r proteins from TNF-stimulated Swiss 3T3 cells

Portions containing the two 43000- M_r proteins and two 41000- M_r proteins were cut from non-alkali-treated gels of Swiss 3T3 cells labelled with [32 P]P_i and treated for 10 min with 20 ng of TNF/ml. Partial acid hydrolysis was performed, and phosphoamino acids were separated on a cellulose plate by electrophoresis (1000 V, 60 min) at pH 3.5 (pyridine/acetic acid/water, 1:10:189, by vol.) as described in the Materials and methods section. Marker phosphoamino acids are phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), identified by ninhydrin staining. Phosphoproteins: 1, pp41-A; 2, pp41-B; 3, pp43-A; 4, pp43-B.

and membrane diacylglycerol, which triggers protein phosphorylation at serine and threonine residues by protein kinase C [22,23]. Thus protein kinase C appears to serve as a signal transducer for a variety of mitogenic agents. The 80000- M_r acidic cellular protein is a major substrate for protein kinase C; increased serine phosphorylation of this protein is believed to be a specific marker *in vivo* of protein kinase C activity [24–26].

To determine the extent of protein kinase C activation in various mitogen-treated Swiss 3T3 cells, we examined the phosphorylation of 80000- M_r protein. TPA induced the markedly increased phosphorylation of 80000- M_r protein; the 80000- M_r phosphoprotein appeared to have multiple isoelectric points, and that from TPA-treated cells predominantly contained more basic forms compared with that from control cells (Figs. 5a and 5d). PDGF and bombesin also stimulated the phosphorylation of 80000- M_r protein; both mitogenic-stimulations generated more basic forms, as did TPA (Figs. 5c and 5d). Acidic forms of 80000- M_r phosphoprotein (pp80-A) from control cells or PDGF-treated cells, and basic forms from TPA- or PDGF-treated cells, were excised and subjected to peptide-map analysis after limited proteolysis with *Staph. aureus* V8 protease. As shown in Fig. 6(a), pp80-A and pp80-B from control or mitogen-treated cells had similar phosphopeptide patterns, with two major fragments of M_r 17600 and 16400. The relative intensity of these two fragments quantified by scanning the autoradiographs on a leser densitometer, however, was somewhat different, about 9:11 for pp80-A and 3:2 for pp80-B. Phosphoamino acid analysis of pp80-A and pp80-B from PDGF-treated cells showed phosphoserine as a major one, and pp80-A contained, in addition, a small amount of phosphothreonine, but pp80-B did not (Fig. 6b). The 80000- M_r phosphoprotein has been reported to contain multiple phosphorylation sites [35], and thus pp80-B generated

by mitogen stimulation may represent phosphoproteins with less phosphate residues on different sites as compared with those in control cells (pp80-A). Down-regulation of protein kinase C by prolonged exposure of Swiss 3T3 cells to TPA completely prevented the subsequent stimulation of 80000- M_r protein phosphorylation by TPA (Fig. 5f), suggesting the involvement of protein kinase C in the phosphorylation of the 80000- M_r protein described above. TNF did not induce increased phosphorylation of 80000- M_r protein at all (Fig. 5b).

DISCUSSION

TNF induces a wide variety of biological effects relevant to regulating cell growth and differentiation which are mediated by the binding of TNF to specific receptors. Although post-receptor mechanisms have been suggested to control the tissue-specific responses of cells to TNF, little is known about the exact molecular basis of them. TNF has recently been shown to induce rapid and transient expression of *c-fos* and *c-myc* in quiescent FS-4 cells [36], which is an early and common transcriptional event occurring in response to stimulation by many growth factors (for reviews, see refs. [37,38]). In the present work, we have shown that TNF rapidly induces the tyrosine phosphorylation of 41000- M_r and 43000- M_r cytosol proteins in mouse and human fibroblasts. Increased tyrosine phosphorylation of those proteins is commonly observed when quiescent fibroblasts are stimulated with a variety of mitogenic agents such as EGF, FGF and PDGF, and is believed to play an important role in the mitogenic signalling pathways of them [19–21]. On the other hand, TNF does not stimulate the serine phosphorylation of an 80000- M_r acidic protein, known to be a major substrate for protein kinase C [24–26]. Mitogenic signalling pathways of growth factors can be classified into two groups, as regards the involvement of protein kinases: one which includes the activation of tyrosine kinase(s), and one which includes the activation of protein kinase C. Our present results clearly show that the mitogenic signalling pathway of TNF includes the activation of tyrosine kinase(s), but not of protein kinase C. In contrast, bombesin seems to transduce its mitogenic signal mainly through the activation of protein kinase C, whereas the activation of both kinases seems to be involved in the mitogenic signalling pathway of PDGF. The possible activation of protein kinase C by TNF has recently been suggested, leading to increased serine phosphorylation of cytosol 26000- M_r protein in U937 cells, which seems to play a role in the TNF signalling pathway linked to differentiation processes rather than to growth control [39].

The precise mechanism by which TNF induces the tyrosine phosphorylation of 41000- M_r and 43000- M_r proteins remains to be clarified. Although specific binding of TNF to the ~80000- M_r polypeptide in many cell types has been reported [40,41], the exact molecular nature of TNF receptors is unclear, and it is unknown whether the TNF receptor itself constitutes an intracellular domain with kinase activity. In this context, it is noteworthy that the increased level of TNF-induced tyrosine phosphorylation of 41000- M_r and 43000- M_r proteins correlates well with the extent of TNF-stimulated DNA synthesis, as in the cases of EGF, FGF and PDGF, but different from those of bombesin and bradykinin (Table 1 and Fig. 2); the receptor molecules for EGF, FGF and PDGF contain intrinsic protein kinase activity specific for tyrosine [18]. TNF has been reported to increase the number of EGF receptors, which may be related to the mitogenic action of TNF in FS-4 cells [42]. Here we have shown that TNF stimulates the tyrosine phosphorylation of 41000- M_r and 43000- M_r proteins of Swiss 3T3 cells in a chemically defined serum-free medium in the absence of EGF. Also, TNF-induced phosphorylation appears as rapidly as in

Fig. 5. Phosphoproteins of Swiss 3T3 cells

Growth-arrested Swiss 3T3 cells were labelled with [32 P]P_i, then treated for 10 min with no addition (a), 20 ng of TNF/ml (b), 20 nM-bombesin (c), 10 ng of PDGF/ml (d) and 100 ng of TPA/ml (e). Swiss 3T3 cells were pretreated with 200 ng of TPA/ml for 24 h, a procedure known to down-regulate protein kinase C, then stimulated for 10 min with 100 ng of TPA/ml (f). Under this condition, TPA did not stimulate DNA synthesis of the cells at all, as determined by the percentage of BrdU-labelled nuclei (0.2%). Labelled phosphoproteins were analysed by two-dimensional gel electrophoresis. Each gel contained the cell lysates of about 4×10^4 cells. The arrow indicates the position of 80000- M_r protein, and small arrowheads in (d) indicate the positions of the two 43000- M_r and two 41000- M_r proteins, which were easily detected after treatment of the gels with 1 M-NaOH and exposure to film for 6–8 times longer than that shown in this Figure (see Fig. 1). Similar results were obtained in four independent experiments.

EGF-stimulated cells, and is maximal 5 min after the exposure of cells to the mitogen. Thus, an indirect mechanism involving EGF-receptor tyrosine kinase in the phosphorylation of those cellular proteins in TNF-treated cells seems unlikely; rather, it may represent a direct and immediate response to TNF/TNF-receptor binding. It might be, although speculative, that the TNF receptor molecule itself possesses tyrosine kinase activity.

Owing to the very limited amounts of 41 000- M_r and 43 000- M_r proteins in cells, and also to the failure to precipitate them with anti-phosphotyrosine antibody (M. Kohno, unpublished work),

all our efforts to purify those proteins have been unsuccessful; thus their biochemical functions still remain to be elucidated. Their cytosolic location in the cells, as clearly shown in this paper, might suggest that they interact with some cytoskeletal proteins; this possibility requires investigation.

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Fig. 6. Partial proteolytic-peptide map and phosphoamino acid analysis of 80000- M_r protein

(a) Spots corresponding to the [32 P] P_i -labelled pp80-A (1, 2) and pp80-B (3, 4) from control (1; C) PDGF-treated (2, 4; P) and TPA-treated Swiss 3T3 cells (3; T) were excised, inserted in the wells of SDS/polyacrylamide (16%) gels, and overlaid with buffer containing 40 ng of *Staph. aureus* V8 protease as described by Cleveland *et al.* [28]. The arrow indicates the position of the unhydrolysed 80000- M_r protein. (b) Portions containing the [32 P] P_i -labelled pp80-A (1) and pp80-B (2) from PDGF-treated Swiss 3T3 cells were incubated in 5.7 M-HCl for 1 h at 110 °C, and released phosphoamino acids were analysed as described in the legend of Fig. 4.

REFERENCES

1. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. & Williamson, B. D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666–3670
2. Williamson, B. D., Carswell, E. A., Rubin, B. Y., Prendergast, Y. S. & Old, L. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5397–5401
3. Baglioni, C., McCandless, S., Tavernier, J. & Fiers, W. (1985) *J. Biol. Chem.* **260**, 13395–13397
4. Kull, F. C., Jacobs, S. & Cuatrecasas, P. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5756–5760
5. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A. & Shepard, H. M. (1985) *Science* **230**, 943–945
6. Tsujimoto, M., Yip, Y. K. & Vilček, J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7626–7630
7. Dayer, J.-M., Beutler, B. & Cerami, A. (1985) *J. Exp. Med.* **162**, 2163–2168
8. Collins, T., Lapiere, L. A., Fiers, W., Strominger, J. L. & Pober, J. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 446–450
9. Bertolini, D. R., Nedwin, G. E., Bringman, T. S., Smith, D. D. & Mundy, G. R. (1986) *Nature (London)* **319**, 516–518
10. Klebanoff, S. J., Vadas, M. A., Harlan, J. M., Sparks, L. H., Gamble, J. R., Agosti, J. M. & Waltersdorff, A. M. (1986) *J. Immunol.* **136**, 4220–4225
11. Nawroth, P. P. & Stern, D. (1986) *J. Exp. Med.* **163**, 740–745
12. Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., Jr. & O'Connor, J. V. (1986) *J. Exp. Med.* **163**, 1433–1450
13. Scheurick, P., Thoma, B., Ucer, U. & Pfizenmaier, K. (1987) *J. Immunol.* **138**, 1786–1790
14. Vilček, J., Palombella, V. J., Henryksen-DeStefano, D., Swenson, C., Feinman, R., Hirai, M. & Tsujimoto, M. (1986) *J. Exp. Med.* **163**, 632–643
15. Beutler, B. & Cerami, A. (1986) *Immunol. Res.* **5**, 281–293
16. Johnson, S. E. & Baglioni, C. (1988) *J. Biol. Chem.* **263**, 5686–5692
17. Hunter, T. (1987) *Cell* **50**, 823–829
18. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930
19. Cooper, J. A., Sefton, B. M. & Hunter, T. (1984) *Mol. Cell. Biol.* **4**, 30–37
20. Kohno, M. (1985) *J. Biol. Chem.* **260**, 1771–1779
21. Kohno, M. & Pouyssegur, J. (1986) *Biochem. J.* **238**, 451–457
22. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
23. Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
24. Rozengurt, E., Rodriguez-Pena, M. & Smith, K. A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7244–7248
25. Rodriguez-Pena, A. & Rozengurt, E. (1986) *EMBO J.* **5**, 77–83
26. Erusalimsky, J. D., Friedberg, I. & Rozengurt, E. (1988) *J. Biol. Chem.* **263**, 19188–19194
27. Hensel, G., Männel, D., Pfizenmaier, K. & Krönke, M. (1987) *Lymphokine Res.* **6**, 119–125
28. Cleveland, D. W., Fischer, S. G., Kirshner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
29. Cooper, J. A. & Hunter, T. (1982) *J. Cell Biol.* **94**, 287–296
30. Tsujimoto, M. & Vilček, J. (1987) *J. Biochem. (Tokyo)* **102**, 1571–1577
31. Cheng, Y. S. E. & Chen, L. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2388–2392
32. Radke, K., Gilmore, T. & Martin, G. S. (1980) *Cell* **21**, 821–828
33. Glenney, J. (1986) *J. Biol. Chem.* **261**, 7247–7252
34. Brugge, J. S. (1986) *Cell* **46**, 149–150
35. Isacke, C. M., Meisenhelder, J., Brown, K. D., Gould, K. L., Gould, S. J. & Hunter, T. (1986) *EMBO J.* **5**, 2889–2898
36. Lin, J. X. & Vilček, J. (1987) *J. Biol. Chem.* **262**, 11908–11911
37. Müller, R. (1986) *Biochim. Biophys. Acta* **832**, 207–225
38. Kelly, K. & Sieberlist, U. (1986) *Annu. Rev. Immunol.* **4**, 317–338
39. Schütze, S., Scheurick, P., Pfizenmaier, K. & Krönke, M. (1989) *J. Biol. Chem.* **264**, 3562–3567
40. Tsujimoto, M., Feinman, R., Kohase, M. & Vilček, J. (1986) *Arch. Biochem. Biophys.* **249**, 563–568
41. Creasey, A. A., Yamamoto, R. & Vitt, C. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3293–3297
42. Palombella, V. J., Yamashiro, D. J., Maxfield, F. R., Decker, S. J. & Vilček, J. (1987) *J. Biol. Chem.* **262**, 1950–1954

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