Further evidence for the involvement of a phosphoprotein in the respiratory burst oxidase of human neutrophils

Paul G. HEYWORTH and Anthony W. SEGAL

Department of Haematology, Faculty of Clinical Sciences, University College London, 98 Chenies Mews, London WC1E 6HX, U.K.

Phosphorylation of a 47 kDa protein in human neutrophils is induced by phorbol 12-myristate 13-acetate (PMA), opsonized latex beads, fMet-Leu-Phe, calcium ionophore A23187 and fluoride. All of these stimuli activate the specialized microbicidal respiratory burst of neutrophils, and in each case the kinetics of activation correspond with the kinetics of phosphorylation of the 47 kDa protein. Trifluoperazine (50 μ M) and chlorpromazine (100 μ M), inhibitors of calmodulin and protein kinase C, abolish the increase in oxygen consumption and selectively prevent phosphorylation of the 47 kDa protein after PMA stimulation. Treatment of neutrophils with pertussis toxin totally inhibits both superoxide production and phosphorylation of this protein in response to fMet-Leu-Phe, but not in response to PMA, indicating that a GTP-binding protein modulates the fMet-Leu-Phe receptor signal. Phosphorylation of the 47 kDa protein, a phenomenon absent from the neutrophils of subjects with autosomal recessive chronic granulomatous disease, which lack the respiratory burst, appears to be the common trigger for activation of the burst in normal neutrophils.

INTRODUCTION

The phagocytic cells of the immune system, neutrophils, monocytes, macrophages and eosinophils, possess a superoxide-generating oxidase system which is important for the killing and digestion of microbes (Selvaraj & Sbarra, 1966). The mechanisms controlling the activation of this burst of non-mitochondrial respiration are not fully understood, but several lines of evidence suggest a role for protein phosphorylation, and in particular the involvement of protein kinase C. Human and animal neutrophils rapidly phosphorylate a number of proteins when stimulated with PMA, a potent activator both of the respiratory burst and of protein kinase C (Nishizuka, 1984), and temporal and dose-response relationships exist between the phosphorylation of some of these proteins and the activation of superoxide generation (Schneider *et al.*, 1981; White *et al.*, 1984; Okamura *et al.*, 1984; Gennaro *et al.*, 1985). In addition, Cox *et al.* (1985) have recently shown that protein kinase C can substitute for cytosol in a reconstituted system of human neutrophils, in which PMA stimulates superoxide production in a plasma membrane fraction. Stimulation of neutrophils (Wolfson *et al.*, 1985) and neutrophil cytoplasts (Gennaro *et al.*, 1986) by PMA is also associated with a rapid translocation of protein kinase C from the cytosol to the particulate fraction, a phenomenon which has been described in other systems thought to involve activation by this enzyme (Nel *et al.*, 1986). These studies have not however revealed the identity of the specific protein kinase substrate which is the presumed trigger for the oxidase system.

Chronic granulomatous disease is a syndrome characterized by a profound susceptibility to infection, in which there is a complete absence of the respiratory burst (Holmes *et al.*, 1967). The X-linked form of CGD is

caused by the absence of cytochrome b_{-245} from the electron transport chain responsible for the oxidase activity (Segal *et al.*, 1983). In the autosomal recessive subgroup of CGD the cytochrome is normal, but it does not receive electrons when cells are stimulated by PMA, indicating that in these patients the defect is in a proximal component of the electron transport chain or in the activation system. We have recently shown that, after treatment with PMA, neutrophils from subjects with autosomal recessive CGD, but not the X-linked form, fail to phosphorylate one particular protein (Segal *et al.*, 1985). It was proposed that this molecule is essential for activation of the respiratory burst, either as a component or in the regulation of the oxidase, and that failure to phosphorylate it is the primary defect in autosomal recessive CGD.

In the present study we investigated the effect of various activators and inhibitors of the respiratory burst on the phosphorylation of this protein in normal human neutrophils. The results indicate that there is a close relationship between the extent of phosphorylation and the activity of the neutrophil oxidase system. They also raise the possibility that dephosphorylation of this protein is the mechanism by which the system is deactivated.

EXPERIMENTAL PROCEDURES

Purification of cells

Neutrophils were purified from 50 ml of freshly drawn human peripheral blood, containing 5 i.u. of heparin/ml, by dextran sedimentation of erythrocytes and differential centrifugation of the supernatant through Ficoll/sodium metrizoate (Segal & Peters, 1977). After removal of contaminating erythrocytes by hypo-osmotic lysis, the

Abbreviations used: CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; HBS, Hepes-buffered saline; DMSO, dimethyl sulphoxide.

cell pellet was washed once in 0.9% (w/v) NaCl, and twice in HBS consisting of 136 mM-NaCl, 2.7 mM-KCl, 1 mM-MgSO₄, 1 mM-CaCl₂, 5.6 mM-glucose, 0.1 mg of bovine serum albumin/ml and 20 mM-Hepes, pH 7.4 (Cockcroft, 1984), in each case by resuspension and centrifugation for 5 min at 20 °C and 220 g. For experiments in which 20 mM-NaF was used as a stimulus the concentration of CaCl₂ in the HBS was reduced to 0.3 mM to avoid precipitation of CaF₂ (Curnutte *et al.*, 1979). Neutrophils (> 97% pure) were finally resuspended in HBS prewarmed to 37 °C at a density of 1×10^7 cells/ml.

Preparation of stimuli and inhibitors

Latex particles (Difco Laboratories, 0.81 μ m diameter) were opsonized by incubation at 37 °C for 30 min with 35 mg of human Ig/ml in 25 mM-Tricine adjusted to

pH 8.5 with NaOH (Segal & Jones, 1979). After centrifugation for 2 min at 12000 g the particles were washed twice in HBS, and finally resuspended in HBS at 1×10^{10} particles/ml. Stock solutions of PMA, 4 α -PDD, fMet-Leu-Phe and A23187 were prepared in DMSO, and trifluoperazine, chlorpromazine and NaF in distilled water. In no experiment was the final concentration of DMSO more than 0.4% (w/v). Pertussis toxin was provided at a concentration of 290 μ g/ml in 50 mM-Tris/HCl (pH 8.0)/1 M-NaCl, and was stored frozen in portions and thawed immediately before use.

Labelling with ³²P_i and activation of cells

Neutrophil suspensions in HBS were incubated at 37 °C for 60 min in the presence of ³²P_i (12.3 MBq/ml). In experiments with pertussis toxin, cells were first incubated for 30 min with the toxin alone (500 ng/ml), ³²P_i was

Fig. 1. Time-course of neutrophil activation by opsonized latex particles

(a) Scans of autoradiographs. Neutrophils labelled with ³²P_i were stirred with opsonised latex particles (5×10^8 /ml) at 37 °C. Samples were removed and mixed with trichloroacetic acid at the indicated times. The precipitated material was solubilized in SDS sample buffer and the samples were centrifuged for 2 min at 11 600 g. The supernatants were subjected to SDS/polyacrylamide-gel electrophoresis as described in 'Experimental procedures' and the gels were dried, autoradiographed and scanned. The positions and size of the marker proteins are shown at the top, and the 47 kDa protein is indicated by an arrow. (b) Oxygen electrode trace. Cells from the same preparation, but not labelled with ³²P_i, were used for the measurement of oxygen consumption as described in 'Experimental procedures'. The times shown on the trace correspond to the times of sampling in parallel experiments with radiolabelled cells.

then added and the incubation continued for a further 60 min. Portions (0.5–1.0 ml) of the labelled suspensions were transferred to the stirred, thermostatically controlled (37 °C) chamber of an oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). A sample (50 μ l, 5×10^5 cells) was removed and rapidly mixed with 1 ml of ice-cold 10% (w/v) trichloroacetic acid and kept on ice. The stimulus was immediately added to the incubation chamber and further samples (50 μ l) were removed at timed intervals and treated in the same way.

When fluoride was used as the stimulus the duration of the lag phase and the maximum rate of respiration were found to vary greatly from one preparation to another. Therefore in these experiments oxygen consumption was measured with simultaneous sampling of phosphorylated cells through the vent in the chamber stopper. With other stimuli oxygen consumption was measured with the same preparation of cells incubated for 60 min in the absence of $^{32}\text{P}_i$.

In experiments with trifluoperazine and chlorpromazine neutrophils were incubated with $^{32}\text{P}_i$ as described above. Suspensions of phosphorylated cells (250 μ l) were transferred to prewarmed tubes containing the inhibitor or an equal volume of water (2.5 μ l). The tubes were mixed and incubated for 2 min at 37 °C prior to the addition of PMA (final concentration 100 ng/ml), or an equal volume of DMSO. After further incubation, 1.0 ml of ice-cold 12.5% (w/v) trichloroacetic acid was added and the tubes stored on ice.

Measurement of superoxide production

To check the efficacy of treatment with pertussis toxin in blocking fMet-Leu-Phe stimulation of the oxidase system, the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm was measured by using the assay system described by Curnutte *et al.* (1974) with 5×10^5 cells in a final volume of 1 ml.

Sample preparation, electrophoresis and autoradiography

The trichloroacetic acid-precipitable material was centrifuged for 5 min at 4 °C and 11600 *g*, the pellets were washed once in ice-cold 10% (w/v) trichloroacetic acid and solubilized in 50 μ l of electrophoresis sample buffer [2.5% (w/v) SDS, 4.0% (v/v) 2-mercaptoethanol, 1 mM-EDTA, 0.01 M-Tris/HCl, pH 6.8]. The solutions were titrated back to near neutrality with NaOH with 1 μ l of a 0.5% (w/v) Bromothymol Blue solution as indicator. After immersion of the samples in a boiling-water bath for 3 min and the addition of 10 μ l of a 60% (w/w) sucrose solution, the samples were subjected to SDS/polyacrylamide-gel electrophoresis on 0.75 mm thick slab gels. These consisted of a 3.7% (w/v) acrylamide stacking gel and a 10% (w/v) separating gel (cross-linker concentration, 2.5% of total acrylamide) and were run at constant current of 18 mA, according to the method of Laemmli (1970). Molecular mass marker proteins were from Pharmacia. Gels were stained with Coomassie Blue, destained, dried under vacuum and autoradiographed with Fuji RX film between intensifying screens. Autoradiographs were scanned with a Joyce-Loebl Chromoscan 3 using a slit width of 0.05 mm and a 280 nm filter.

Materials

PMA, 4 α -PDD, fMet-Leu-Phe, A23187, trifluoperazine and chlorpromazine were purchased from Sigma. Pertussis toxin was obtained from the Public Health

Laboratory Service, Porton Down, Wilts, U.K., and carrier-free $^{32}\text{P}_i$ (PBS.11) from Amersham International.

RESULTS

Pattern of protein phosphorylation with different stimuli

Only two protein bands, with apparent molecular masses of 47 ± 0.43 kDa and 43 ± 0.48 kDa (mean $\pm$ s.d.

Fig. 2. Protein phosphorylation in human neutrophils stimulated with different concentrations of PMA

Neutrophils labelled with $^{32}\text{P}_i$ were treated with PMA for 3 min at 37 °C. The reaction was stopped with trichloroacetic acid and the precipitated material was subjected to SDS/polyacrylamide-gel electrophoresis as described in 'Experimental procedures'. The autoradiograph of the dried gel is shown. Track A, DMSO control; tracks B, C and D, 100, 10 and 1 ng of PMA/ml respectively. The position and size of the marker proteins are shown and the 47 kDa band is indicated by the arrow.

from 15 tracks on four gels, estimated from standard curves constructed with marker proteins of 94, 67, 43, 30, 20.1 and 14.4 kDa), showed enhanced phosphorylation with all five stimuli used in this study (compare Figs. 1–5). The 47 kDa band corresponds to the protein described by Segal *et al.* (1985), which fails to become phosphorylated in neutrophils from subjects with autosomal recessive CGD. In this previous study we used a slightly different polyacrylamide gel composition (3% stacking gel and 2.6% cross-linker), and although the phosphoprotein appeared to run slightly slower than the 45 kDa standard in some gels (e.g. see Figs. 2 and 4 of Segal *et al.*, 1985), the apparent mean molecular mass was 44.0 ± 1.0 kDa ($\pm$ s.d. from 21 tracks) when estimated from standard curves constructed with only four molecular mass markers (77, 66.7, 45 and 25.7 kDa). In both studies the protein consistently appears in our scans of autoradiographs of normal cells, immediately to the left of a deep trough at approx. 43–45 kDa.

The scans of autoradiographs presented here are, of necessity, from single experiments; in each case similar results, both qualitative and quantitative, have been observed in at least two experiments.

The addition of PMA leads to the enhanced phosphorylation of about ten proteins as previously reported (Schneider *et al.*, 1981; Segal *et al.*, 1985) (Fig. 2); in each case phosphorylation persists for at least 5 min. In the presence of opsonized latex, at a ratio of approx. 50 particles/cell, there was enhanced phosphorylation of fewer proteins than with PMA, which

was transient in the case of the bands at 43 and 47 kDa. Phosphorylation of the 47 kDa band is apparent after 10–20 s and is maximal at about 80 s (Fig. 1).

In dose-response experiments, only concentrations of PMA sufficient to activate the burst (> 2 ng/ml with a cell density of 1×10^7 cells/ml) induced phosphorylation of the 47 kDa protein. Fig. 2 shows the effects of 1, 10 and 100 ng of PMA/ml on the pattern of phosphorylation after 3 min. At the lowest concentration there was no enhancement of phosphorylation in the 47 kDa band, but a strong enhancement in the 43 kDa protein. Exposure of neutrophils to 4α -PDD, at 10 and 100 ng/ml, neither activated the respiratory burst nor led to the enhanced phosphorylation of any protein (results not shown).

Activation of the respiratory burst with 100 nM-fMet-Leu-Phe was characterized by a very short (< 10 s) lag phase, and a short period of fast, linear oxygen consumption followed by a longer period of greatly reduced consumption (Fig. 3). The number of protein bands which showed enhanced phosphorylation on stimulation with fMet-Leu-Phe was small and as with latex, phosphorylation of the 47 kDa band was transient. With the calcium ionophore A23187 the 47 kDa protein was rapidly (< 10 s) but weakly phosphorylated and this persisted for at least 5 min (results not shown).

The stimulatory effect of 20 mM-fluoride on the respiratory burst of human neutrophils was relatively weak, and as previously reported (Curnutte *et al.*, 1979) the lag phase was very long (8–12 min) (Fig. 4).

Fig. 3. Time-course of neutrophil activation by fMet-Leu-Phe

(a) Scans of autoradiograph. Details are as described in the legend to Fig. 1 and in 'Experimental procedures', except that 100 nM-fMet-Leu-Phe was used to stimulate the cells. (b) Oxygen electrode trace. Details as for Fig. 1.

Fig. 4. Time course of neutrophil activation by fluoride

(a) Scans of autoradiograph. Details as for Fig. 1 except that cells were stimulated with 20 mM-NaF and oxygen consumption was recorded throughout the experiment.

(b) Oxygen electrode trace. Times are indicated at which samples of radiolabelled cells were removed.

Treatment of neutrophils with fluoride resulted in phosphorylation of the 43 and 47 kDa protein bands but with very different time courses. Enhanced phosphorylation of the 43 kDa band was apparent after 1 min, maximal at about 8 min and by 12 min had decreased substantially. The lag period for the enhanced phosphorylation of the 47 kDa protein was 8–10 min, corresponding closely to that preceding the onset of the respiratory burst. In control experiments with 20 mM-NaCl there was no detectable effect on neutrophil oxygen consumption and protein phosphorylation.

Effect of inhibitors of the oxidase on the pattern of protein phosphorylation

Pretreatment of neutrophils for 2 min with the calmodulin inhibitors trifluoperazine (50 μ M) and chlorpromazine (100 μ M), both of which are also known to

inhibit protein kinase C (Nishizuka, 1984), abolished PMA stimulation of the respiratory burst and specifically inhibited PMA-induced phosphorylation of the 47 kDa protein (Fig. 5). Phosphorylation of all other bands remained unaffected.

fMet-Leu-Phe-mediated neutrophil functions are thought to be modulated by a GTP-binding protein (Koo *et al.*, 1983), whereas PMA is believed to act directly on protein kinase C. Incubation of neutrophils for 90 min with 500 ng of pertussis toxin/ml resulted in the total inhibition of fMet-Leu-Phe-stimulated phosphorylation of the 47 kDa protein (Fig. 6). The PMA-stimulated pattern of protein phosphorylation was unaffected. Pertussis toxin also blocked superoxide production in response to fMet-Leu-Phe but not that stimulated by PMA (Fig. 6).

DISCUSSION

We have shown that the 47 kDa protein, which is phosphorylated in intact normal neutrophils stimulated with PMA, but remains unphosphorylated in neutrophils from patients with autosomal recessive CGD (Segal *et al.*, 1985), is also phosphorylated when the respiratory burst of normal cells is activated with other agents. The extent and time-dependency of phosphorylation differs with the stimulus used, but none that we have tested was able to induce a burst in the absence of phosphorylation of this protein.

PMA, which produces a prolonged burst of oxygen consumption, induces the persistent, enhanced phosphorylation of about ten protein bands, as previously demonstrated (Segal *et al.*, 1985). However, pretreatment of cells for 2 min with either trifluoperazine or chlorpromazine, inhibitors of the calcium-binding protein calmodulin and of protein kinase C (Nishizuka, 1984), at concentrations just sufficient to inhibit the respiratory burst, specifically inhibit PMA-induced phosphorylation of the 47 kDa protein (Fig. 5). The specificity of inhibition implies that of the proteins phosphorylated, this molecule alone is intimately involved in activation of the oxidase.

If the concentration of PMA is reduced to levels too low to activate the oxidase (< 2 ng/ml), phosphorylation of the 47 kDa band is not observed (Fig. 2). However, at these sub-threshold doses the 43 kDa protein is still heavily phosphorylated. This band is also phosphorylated normally in autosomal recessive CGD (Segal *et al.*, 1985) and it is not inhibited by trifluoperazine and chlorpromazine, indicating that although possibly involved in neutrophil activation, it is not the direct trigger for the oxidase system.

The varied responses of neutrophils to fMet-Leu-Phe, such as chemotaxis, secretion and superoxide production, are believed to be modulated by a GTP-binding protein (Koo *et al.*, 1983; Lad *et al.*, 1985), analogous to N_i of the adenylate cyclase system (Cockcroft, 1986) that couples the fMet-Leu-Phe receptor to polyphosphoinositide phosphodiesterase (Verghese *et al.*, 1985). Treatment with pertussis toxin blocks the neutrophil responses to fMet-Leu-Phe, presumably by catalysing NAD-dependent ADP-ribosylation of the GTP-binding protein, but has no effect on neutrophil activation by PMA (Goldman *et al.*, 1985; Verghese *et al.*, 1985; Volpi *et al.*, 1985; Barrowman *et al.*, 1986). As pertussis toxin affects neither the number nor the affinity of fMet-Leu-Phe

Fig. 5. Effect of inhibitors on the respiratory burst and protein phosphorylation in neutrophils

(a) Scans of autoradiographs. Neutrophils labelled with $^{32}\text{P}_i$ were preincubated for 2 min with no inhibitor (i), 50 μM -trifluoperazine (TFP) (ii) or 100 μM -chlorpromazine (CP) (iii). PMA (100 ng/ml, upper trace of each pair) or an equal volume of DMSO (lower trace) were added and the reaction was stopped with trichloroacetic acid after a further 2 min incubation. Following SDS/polyacrylamide-gel electrophoresis of the precipitated material, the dried gels were autoradiographed and scanned. The positions of the marker proteins are shown at the top of the figure, and the 47 kDa band is indicated by the arrow. (b) Oxygen electrode traces. Details as for Fig. 1. Cells were preincubated for 2 min with the inhibitors before the addition of 100 ng of PMA/ml.

Fig. 6. Effect of pertussis toxin treatment on protein phosphorylation and superoxide production in neutrophils

(a) Scans of autoradiographs. Labelling with $^{32}\text{P}_i$ and treatment with pertussis toxin was as described in 'Experimental procedures'. Untreated cells (i and ii) and treated cells (iii and iv) were incubated for 20 s with activators (i and iii, 100 ng of PMA/ml; ii and iv, 100 nM-fMet-Leu-Phe; upper trace of each pair) or DMSO (lower traces). Trichloroacetic acid precipitates were electrophoresed as previously described and the dried gels were autoradiographed and scanned. (b) Traces show the rate of superoxide production by pertussis-treated and untreated cells stimulated with PMA or fMet-Leu-Phe (key as for a), as described in 'Experimental procedures'.

binding sites (Volpi *et al.*, 1985), its ability to inhibit phosphorylation of the 47 kDa protein in fMet-Leu-Phe-treated neutrophils is presumably due to its action on the GTP-binding protein. Therefore in neutrophils stimulated with fMet-Leu-Phe, phosphorylation of the 47 kDa protein is possibly a consequence of receptor-mediated activation of polyphosphoinositide phosphodiesterase and the resulting transient increase in diacylglycerol, the physiological activator of protein kinase C (Nishizuka, 1984). Alternatively phosphorylation could be catalysed by another calcium-dependent protein kinase, activated by the rapid increase in intracellular Ca^{2+} , caused in turn by the release of inositol trisphosphate from phosphoinositide. Whatever the mechanism, phosphorylation is relatively weak and transient; dephosphorylation appears to precede the decrease in the rate of oxygen consumption, raising the possibility that the oxidase is switched off by a protein phosphatase.

The fluoride anion has long been recognized as an activator of the respiratory burst of neutrophils (Sbarra & Karnovsky, 1959), although its mode of action is unknown (Curnutte *et al.*, 1979). Our observation that fluoride induces phosphorylation of the 47 kDa protein (Fig. 4) suggests two possibilities. The first is that the non-specific inhibitory action of fluoride on protein phosphatases (Pato & Adelstein, 1983) eventually leads to the accumulation of a sufficiently high level of phosphorylation of the protein to trigger the burst. The second and perhaps more likely possibility is that fluoride activates the GTP-binding (G-) protein transducing the signal from the fMet-Leu-Phe receptor, in a manner analogous to fluoride activation of N_1 in the adenylate cyclase system. This mechanism has been proposed for fluoride-induced mobilization of intracellular calcium in human neutrophils (Strnad & Wong, 1985) and these authors suggest that the long lag phase (4–10 min for calcium mobilization) is due to the time taken by the fluoride anion to reach its site in the membrane. As discussed above, the rise in intracellular calcium may activate a calcium-dependent protein kinase for which the 47 kDa protein is a substrate.

It is possible that the phosphoprotein we have identified is the same molecule described by other workers in animal neutrophils. A cytosolic polypeptide of 46 kDa is phosphorylated in bovine neutrophil cytoplasts stimulated with PMA, latex beads and ionomycin (Gennaro *et al.*, 1985) and Okamura *et al.* (1984) describe two proteins of this size that become phosphorylated in rabbit neutrophils exposed to membrane perturbants. White *et al.* (1984) have shown that a protein of about 50 kDa is phosphorylated in rabbit peritoneal neutrophils stimulated with fMet-Leu-Phe or PMA, and that fMet-Leu-Phe-induced phosphorylation of the protein is inhibited by more than 70% in pertussis toxin-treated cells (Volpi *et al.*, 1985). Cross & Jones (1986) have recently reported on a polypeptide present in the solubilized oxidase of pig neutrophils which binds diphenylene iodonium, an inhibitor of the oxidase.

Several authors have commented on the differences between the activation of the neutrophil respiratory burst by PMA, latex particles and fMet-Leu-Phe, particularly with respect to the kinetics of oxygen consumption and the effect of inhibitors (McPhail & Snyderman, 1983; Cooke & Hallett, 1985; Gerrard *et al.*, 1986; Cockcroft, 1986). This has led to the proposal that two distinct

mechanisms exist; one depending on direct activation of protein kinase C and the other involving receptor occupancy and G-protein-modulated changes in phosphatidylinositol metabolism. Our findings that the kinetics of phosphorylation of the 47 kDa protein depend on the stimulus used, and that pertussis toxin treatment selectively inhibits fMet-Leu-Phe-induced phosphorylation, support the existence of parallel pathways. However, they also indicate that all inducers of the burst must act via the 47 kDa protein, possibly by stimulating different protein kinases. This view is supported by the fact that neutrophils from subjects with autosomal recessive CGD fail to generate a respiratory burst irrespective of the stimulant used (Hitzig & Seger, 1983). Assuming that failure to phosphorylate the 47 kDa protein is the single, primary defect in this subgroup of CGD (Segal *et al.*, 1985) the 47 kDa protein could well be the common trigger through which the different stimuli ultimately act.

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