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INTRODUCTION

Cells must continuously adapt to external stimuli. The molecular details of most of the underlying signalling pathways have now been partially clarified and frequently involve the sequential phosphorylation of the molecules involved. One well-characterized cascade is the signalling pathway initiated by active p21 Ras leading to the sequential activation of Raf, mitogen-activated protein kinase (MAPK), p42/p44 MAPK [1–4]. Other signalling cascades, analogous to this pathway, lead to the phosphorylation and stimulation of p38 MAPK and stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), which both show significant sequence homology to p42/p44 MAPK. Aberrant control of these signal-transduction pathways has been implicated in a variety of pathological conditions and is therefore an interesting target for both drug discovery and research into the alterations of signal transduction in primary material isolated from individual patients.

Unfortunately, current methods for assaying activation of p38 MAPK, p42/p44 MAPK or SAPK/JNK, such as Western blotting and gel-based kinase assays, are labour-intensive and do not allow the study of large numbers of samples. The situation has improved recently with the advent of phosphospecific antibodies, which recognize the phosphorylated forms of proteins but not their unphosphorylated counterparts. However, analysis by Western blotting and gel-based kinase assays remains dependent on kinase assays or Western blotting. Similarly, cAMP-dependent transactivation of cAMP-response-element-containing promoters through protein kinase A and phosphorylation of cAMP-response-element-binding protein (CREB) [8,9] remains a difficult target for high-throughput screening.

We therefore developed an ELISA-based assay to measure the activation of specific signalling pathways by employing phosphospecific antibodies, which recognize only the phosphorylated (activated) state of signalling components. We used these antibodies to determine immunoreactivity of cells grown in 96-well plates exposed to various stimuli and we have thus created the phosphospecific antibody cell-based ELISA (PACE). PACE and similar ELISA-based techniques are non-radioactive semi-quantitative assays of signal-transduction pathways that are suited for the investigation of large numbers of samples and allow high-throughput screening. We suggest that the assessment of signal transduction by PACE will become the technique of choice for rapid analysis.

MATERIALS AND METHODS

Materials and cell culture

N-Formylmethionyl-leucylphenylalanine (fMLP), poly-l-lysine, o-phenylenediamine dihydrochloride (OPD) and BSA were from Sigma (St. Louis, MO, U.S.A.). Human serum albumin was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). The polyclonal antibody against p44/42 MAPK was from UBI (Lake...
Placid, NY, U.S.A.) and polyclonal anti-phospho-p44/42 MAPK, anti-phospho-p38 MAPK, anti-phospho-c-Akt/PKB and anti-phospho-CREB antibodies were from New England Biolabs (Beverly, MA, U.S.A.), as were all in vitro kinase-assay kits. Pig anti-rabbit peroxidase-conjugated antibody and goat anti-rabbit antibody were from Dako. PD98059 was from Biomol (Plymouth Meeting, PA, U.S.A.). Murine macrophages clone 4/4 [10] were cultured according to routine procedures. Blood was obtained from healthy volunteers. Mixed granulocytes were isolated as described earlier [11].

**Cell-based ELISA for adherent cells**

Cultured murine macrophages were seeded in 96-well plates (Maxisorp, Nunc) at 50000 cells/cm². When appropriate, the cells were serum-starved for 4 h and stimulated for 10 min. After stimulation, the cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100 (hereafter referred to as PBS/Triton). Endogenous peroxidase was quenched with 0.6% H₂O₂ in PBS/Triton for 20 min, washed three times in PBS/Triton, blocked with 10% fetal calf serum in PBS/Triton for 1 h and incubated overnight with various dilutions of primary antibody in PBS/Triton containing 5% BSA at 4°C. Next day, cells were washed three times with PBS/Triton for 5 min and incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit antibody, dilution 1:100) in PBS/Triton with 5% BSA for 1 h at room temperature and washed three times with PBS/Triton for 5 min and twice with PBS. Subsequently the cells were incubated with 50 μl of a solution containing 0.4 mg/ml OPD, 11.8 mg/ml Na₃HPO₄·2H₂O, 7.3 mg/ml citric acid and 0.015% H₂O₂ for 15 min at room temperature in the dark. The reaction was stopped with 25 μl of 1 M H₂SO₄, the A₄90-650 was measured and S.E.M. values were determined (see the error bars in Figures).

**Cell-based ELISA for non-adherent cells**

A 96-well plate was coated with 10 μg/ml poly-l-lysine for 30 min at 37°C. After two washes with sterile PBS, 1.5 x 10⁵ granulocytes were plated into each well and incubated for 30 min at 37°C. Granulocytes were stimulated with fMLP and fixed with 8% formaldehyde in PBS for 20 min at room temperature, followed by three washes with PBS/Triton. Quenching was performed with 1% H₂O₂ and 0.1%, sodium azide in PBS/Triton for 20 min and cells were treated further as described for adherent cells with the exception of the use of a 1:500 dilution of the secondary antibody (peroxidase-conjugated pig anti-rabbit) in PBS/Triton with 5% BSA for 1 h at room temperature.

**Crystal violet cell-quantification assay**

After the peroxidase reaction, the cells were washed twice with PBS/Triton and twice with demineralized water. After drying the wells for 5 min, 100 μl of crystal violet solution [0.04%, crystal violet in 4% (v/v) ethanol] was added for 30 min at room temperature. Subsequently, the cells were washed at least three times with demineralized water and 100 μl of 1%, SDS solution was added and incubated on a shaker for 1 h at room temperature. Finally, the absorbance was measured at 595 nm with an ELISA reader.

**In vitro kinase assays**

Murine macrophages were grown in either 6-well plates (p42/p44 MAPK assay) or 60-mm dishes (p38 MAPK and PKB assays) and serum starved for 16 h. After appropriate stimulation cells were treated according to the manufacturer of the assay kits (New England Biolabs).

**Western blotting**

Cell lysate or kinase reaction mix (both 40 μl) was loaded on to SDS/polyacrylamide gels and blotted on to PVDF membranes. After blocking and washing, bands were visualized using the appropriate phosphospecific antibodies (1:1000), peroxidase-conjugated goat anti-rabbit antibody (1:2000) and enhanced chemiluminescence.

**RESULTS AND DISCUSSION**

**Development of a p42/p44 MAPK phosphorylation assay in 96-well plates**

Activation of p42/p44 MAPK is accomplished by phosphorylation of the protein on threonine¹⁸⁵/tyrosine¹⁸⁵ and threonine²⁰²/tyrosine²⁰⁴ by MEK [4], and commercial antibodies recognizing phosphorylated p42/p44 MAPK with little cross-reactivity to

**Figure 1 Results of a p42/p44 MAPK PACE**

(A) Clone 4/4 macrophage cells were grown in a 96-well plate, stimulated with the indicated concentrations of PMA (TPA) and subjected to PACE procedures. Results obtained with different dilutions of the polyclonal anti-phospho-p42/p44 MAPK antibody as primary antibody are shown. Phosphorylation and activity of MAPK was determined by analysis on Western blot (B) and an in vitro kinase assay (C). ERK, extracellular-signal-regulated kinase.
Figure 2  fMLP-mediated p42/p44 MAPK activation in neutrophils measured by PACE

Human neutrophils were isolated from peripheral blood, adhered to poly-L-lysine-coated 96-well plates and stimulated for 2 min with fMLP (○) or PD98059 and fMLP (●; left-hand panel). The right-hand panel shows the effects of 10⁻⁷ M fMLP for different time periods. After stimulation p42/p44 MAPK PACE was performed. ERK, extracellular-signal-regulated kinase.

Figure 3  Results of a p38 MAPK PACE

(A) Clone 4/4 cells were grown in a 96-well plate and stimulated with lipopolysaccharide (LPS), followed by PACE procedures. Results obtained with different dilutions of a polyclonal anti-phospho-p38 MAPK antibody are shown. Analysis of p38 MAPK by Western blotting (B) and an in vitro kinase assay (C) are also shown. ATF, activating transcription factor.

the unphosphorylated form of MAPK or other cellular proteins are available (Figure 1). We analysed how these antibodies would perform when used for an ELISA-based assay. Therefore, clone 4/4 macrophages were challenged with different concentrations of PMA, a potent activator of MAPK in these cells. Subsequently, the cells were fixed, blocked, washed (see the Materials and methods section) and incubated with different concentrations of anti-phospho-p42/p44 MAPK antibody followed by further routine ELISA procedures, finally culminating in oxidation of OPD by a peroxidase reaction and determination of absorbance at 490/650 nm. We have termed this procedure PACE. As evident from Figure 1, in unstimulated cells or in the absence of primary or secondary antibody, little enzymic activity is detected. Increasing concentrations of PMA, however, yield increased levels of enzymic product (Figure 1A), the dose–response curve observed in the ELISA being in strict accordance with the dose–response curve of PMA-enhanced MAPK activity, as observed with Western blots or in vitro kinase assays (Figures 1B and 1C). We found that a 1:250 dilution of anti-phospho-p42/p44 MAPK combined low background with high sensitivity. The specificity of PACE was established further by the observation that PD98059, which inhibits activation of MEK [12], blocked the PMA-induced increase in absorbance (results not shown). We concluded that PACE is a sensitive and specific method for determining p42/p44 MAPK activation.

PACE is useful for primary isolated suspension cells

In the PACE described above, the immunoreactive substance, i.e. phosphorylated p42/p44 MAPK, is immobilized via formaldehyde fixation of cells grown in 96-well tissue-culture plates. To adapt the assay for cell types which do not naturally attach to tissue-culture plastic, we adhered primary isolated human granulocytes to poly-L-lysine-coated ELISA plates. When tested in PACE according to the procedures employed for clone 4/4 macrophages we encountered a high background signal due to endogenous peroxidase activity. Inclusion of 0.1% sodium azide was sufficient to quench this activity. Concentration- and temporal-dependent, PD98059-sensitive, fMLP-induced enzymic product formation was readily detected (Figure 2). The p42/p44 MAPK activation by fMLP closely matched that observed in primary isolated human granulocytes using classical kinase assays or Western-blot analysis [11]. Thus PACE is a specific and sensitive method for detection of p42/p44 MAPK phosphoryl-
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Figure 4 Results of a c-Akt/PKB PACE

Clone 4/4 cells grown in a 96-well plate were stimulated with insulin (Ins) followed by PACE procedures. (A) Results with different concentrations of an anti-phospho-c-Akt/PKB antibody are shown. The effect of wortmannin (Wm) on insulin-stimulated cells was also determined (right-hand panel). Phosphorylation and activity of PKB determined by Western blotting (B) and in vitro kinase assay (C) are also depicted. GSK, glycogen synthase kinase.

Development of a PACE for p38 MAPK, c-Akt/PKB and CREB

We investigated whether phosphospecific antibodies directed against other signal-transduction components are applicable for PACE. Macrophages were challenged with different concentrations of lipopolysaccharide, a potent activator of p38 MAPK. Subsequently, the cells were fixed and incubated with different concentrations of anti-phosphothreonine\(^{180}\)/phosphotyrosine\(^{182}\) p38 MAPK antibody, followed by further routine ELISA procedures. Again little enzymic product formation was detected in the absence of either the primary or secondary antibody or in the absence of lipopolysaccharide. Increasing concentrations of lipopolysaccharide, however, produced a dose-dependent increase in immunoreactivity (Figure 3A). We found that an anti-phospho-p38 MAPK antibody dilution of 1:250 combined low background with high sensitivity. The PACE-detected increase in phosphorylated p38 MAPK corresponded to that observed using Western blotting and to activity in in vitro kinase assays (Figure 3C). Although the phosphospecific p38 MAPK antibody is reported to have cross-reactivity with other stress-activated MAPKs, we only detected one band on the Western blot.

We also tested a phosphoserine\(^{171}\)-specific c-Akt/PKB antibody in PACE. Macrophages were stimulated with the PKB activator insulin. As shown in Figure 4(A), we observed a dose-dependent stimulation of enzymic product formation, in agreement with the insulin-dependent increase of c-Akt/PKB activity observed in both Western blotting (which also showed the specificity of the antibody for c-Akt/PKB; Figure 4B) and in vitro kinase assays (Figure 4C). The optimal dilution of primary antibody (1:100) was higher than those observed with the p42/p44 MAPK and p38 MAPK PACE. The specificity, however, of the c-Akt/PKB PACE was confirmed by the sensitivity of the insulin-induced signal to wortmannin. Finally we tested a phosphoserine\(^{172}\)-specific CREB antibody in PACE. To this end clone 4/4 macrophages were stimulated with prostaglandin E\(_2\), prostaglandin F\(_{2\alpha}\) and prostaglandin I\(_2\), since various prostaglandins have been reported to mediate CREB phosphorylation [13–16], but the exact effects of these important inflammatory mediators on CREB phosphorylation in macrophages have not yet been assessed. As shown in Figure 5(A), prostaglandin E\(_2\) resulted in CREB phosphorylation in macrophages as detected by Western blotting (the antibody also cross-reacts with activating transcription factor 1, ATF1). After stimulation, a CREB PACE was performed, whereas cAMP production was measured in a parallel experiment. Prostaglandin F\(_{2\alpha}\) did not induce cAMP production nor did it lead to the production of enzymic product.
Figure 5  Effects of prostaglandin treatment on cAMP production and CREB

(A) Clone 4/4 cells were grown in 60-mm dishes and stimulated with prostaglandin E₂ (PGE₂). CREB phosphorylation was subsequently measured by Western blotting, using phosphospecific CREB antibodies. (B) Effects of PGE₂, PGI₂ and PGF₂α on cAMP production and CREB phosphorylation, measured using a commercial enzyme-linked immunoassay kit for cAMP (Amersham) and PACE. ATM, activating transcription factor.

In a PACE assay (Figure 5B). In contrast, prostaglandin E₂ and prostaglandin I₂ gave rise to a significant increase in cAMP production and CREB immunoreactivity in PACE. Increases in cAMP formation did not completely correlate with increases in CREB phosphorylation at lower concentrations of prostaglandins, but this was attributed to cAMP-independent phosphorylation of CREB [14]. We concluded that PACE is a convenient method to determine the phosphorylation state of
employed may react with other cellular proteins. This is not a
major problem if this antibody reactivity does not differ between
stimulated and unstimulated cells, as this will only result in some
additional background signal. If, however, immunoreactivity is
induced by stimulation of the cells, a problem may arise. Western
blotting of PMA-stimulated cells using the anti-phospho-p42/
p44 MAPK antibody reveals, after prolonged exposure, some
faint induced bands apart from the immunoreactivity at 42 and
44 kDa, and these bands may contribute to the signal measured
in the ELISA. Nevertheless, the enhanced chemiluminescence
used to detect antibody binding is not a very linear technique
(meaning that an increase in the amount of immunoreactive
material does not correspond closely with the increase in obtained
signal), is very sensitive and saturates quickly. The contribution
of phospho-p42/p44 MAPK to the signal measured in PACE
may therefore be even greater than estimated from the Western
blot. Indeed, one of the major advantages of PACE over
Western blotting is that the enzymic reaction underlying PACE
is of a more linear nature and therefore produces quantitatively
more accurate results.

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Figure 6 Correcting for differences in cell number by staining the wells
with crystal violet after the PACE procedure

Clone 4/4 cells were stimulated with PMA (TPA) and subjected to PACE (upper panel). Subsequently cell numbers in individual wells were determined using crystal violet staining and measuring absorbance at 595 nm, and the ratios were calculated (lower panel). A, Background signal in the absence of primary antibody; B, background in the absence of secondary antibody; C, background in the absence of both primary and secondary antibody. Note the reduction in S.E.M. by correcting for differences in cell number.

Correcting for differences in cell number

The validity of the PACE assay critically depends on two factors: the loading of equal amounts of cells in different wells and the quality of the primary antibody. We investigated, therefore, whether it is possible to correct for the former factor using crystal violet staining after the PACE procedure. As shown in Figure 6, crystal violet staining works well under these conditions and provides the opportunity to correct the signals measured in PACE for cell number, as evident by the reduction in variability in the assay. With respect to the latter factor, the antibodies employed may react with other cellular proteins. This is not a

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