cAMP suppresses p21^{ras} and Raf-1 responses but not the Erk-1 response to granulocyte-colony-stimulating factor: possible Raf-1-independent activation of Erk-1

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The cAMP analogue 8-bromo-cAMP (8BrcAMP) inhibits granulocyte-colony-stimulating factor (G-CSF)-stimulated DNA synthesis in myeloid NFS-60 cells. We examined the effect of 8BrcAMP addition on the G-CSF-stimulated extracellular signal-related protein kinase 1 (Erk-1), p21^{ras} and Raf-1 activation. The Erk-1 activity was not down-regulated by the increase in intracellular cAMP levels, whereas p21^{ras} and Raf-1 activities were, suggesting that Erk-1 activity might not be dependent on upstream p21^{ras} and/or Raf-1 activity in this system. To explore this possibility further, we sought to determine whether there were downstream substrates of Raf-1 that were distinguishable from those of Erk-1 by using two-dimensional SDS/PAGE analysis of the protein phosphorylation patterns of NFS-60 cell cytosolic extracts treated with exogenous Raf-1 or Erk-1 in the presence of [γ -³²P]ATP. The two phosphorylation patterns were

found to have many differences. To gain further insights into the possible relevance of these phosphorylation patterns and as an approach to exploring in more detail the inhibitory effect of 8BrcAMP, two-dimensional SDS/PAGE analysis was performed on the cytosolic extracts of ³²P-labelled NFS-60 cells treated with G-CSF, in the absence or presence of 8BrcAMP. It was found that the phosphorylated proteins whose appearance was specific to the action of exogenous Raf-1 were sensitive to the action of 8BrcAMP *in vivo*, whereas those whose appearance was specific to the action of exogenous Erk-1 alone, or common to the actions of Raf-1 and Erk-1, were 8BrcAMP-insensitive. The results are consistent with a Raf-1-independent pathway for Erk-1 activation in G-CSF-treated myeloid cells, and a number of potential downstream substrates of these kinases have been identified for further characterization.

INTRODUCTION

Granulocyte-colony-stimulating factor (G-CSF) controls the proliferation, differentiation and function of cells along the granulocyte lineage and is used clinically to restore the neutrophil count in patients undergoing cancer therapy [1,2]. The effects of G-CSF are mediated after binding to its gp130-related homodimeric receptor [3,4]. The signal transduction pathways utilized by this interaction have recently begun to be examined.

G-CSF has been shown to activate p21^{ras} and mitogen-activated protein kinase (MAPK) in myeloid NFS-60 cells [5]; similarly, BAF/BO3 cells, transfected with the human G-CSF receptor, have been reported to exhibit a G-CSF-dependent activation of MAPK [6]. In general the activation by cytokines of the MAPK family members Erk-1 (p44^{MAPK}) and Erk-2 (p42^{MAPK}) is believed to be mediated by the action of MAPK kinase (MEK) [7], which is itself activated by either Raf-1 [8–10], MEK kinase [11] or Mos [12]. It is usually assumed that Raf-1 in turn is activated by its recruitment to the Sos/Grb2/cytokine receptor complex via the action of GTP-p21^{ras} [13]. However, there is evidence for p21^{ras}-dependent and -independent activation of MAPK [14,15]. Which aspects of these pathways are relevant to G-CSF-mediated signalling has yet to be elucidated.

Increased intracellular cAMP levels can result in either enhanced proliferation or growth suppression depending on the

cell type. Hepatocytes, epithelial cells and Swiss 3T3 cells proliferate after the elevation of intracellular cAMP levels [16–18]. In contrast, cAMP inhibits proliferation in lymphoid cells, epidermal growth factor-treated fibroblasts, platelet-derived growth factor-treated smooth muscle cells, macrophage colony-stimulating factor (M-CSF or CSF-1)-treated macrophages and G-CSF-stimulated myeloid cells [19–23]. The signal transduction pathways involved in the negative control of cell proliferation by elevated cAMP are not completely defined. A number of reports have demonstrated that increasing intracellular cAMP inhibits MAPK activity, via the phosphorylation of Raf-1 by protein kinase A, which prevents its activation by p21^{ras} [24–29]. However, there are reports suggesting that suppression of MAPK activity does not always occur in cells whose growth is suppressed by elevated intracellular cAMP [15]. In cases where cAMP elevation results in enhanced proliferation, MAPK activity can be stimulated [15,30].

Because cAMP activates protein kinase A, an analysis of the protein phosphorylation changes can be used to define the targets of this kinase family. Also, if increased cAMP levels can suppress growth factor-mediated proliferation, then this intracellular messenger might be used to dissect the signal transduction pathways governing such proliferation, particularly if protein phosphorylation changes are monitored. By using one-dimensional SDS/PAGE, a number of uncharacterized phospho-

Abbreviations used: 8BrcAMP, 8-bromo-cAMP; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular signal-related protein kinase; FBS, foetal bovine serum; G-CSF, granulocyte-colony-stimulating factor; G-CSFR, G-CSF receptor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase.

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proteins have been observed after G-CSF treatment of various cell types [2,31,32]. The significance and function of these phosphoproteins, including their novelty and specificity for G-CSF signalling, remain to be determined. Two-dimensional SDS/PAGE is now a more reproducible technique owing to accumulated protocol modifications, standardization of procedures and materials, increased purity of ampholytes and greater commercial input into hardware. Because of the greater resolving power of two-dimensional SDS/PAGE, we have recently been able to detect a number of ^{32}P -labelled proteins whose phosphorylation status is altered specifically after G-CSF treatment [32a].

In this study we have used two-dimensional SDS/PAGE to delineate a number of cytosolic phosphoproteins in a G-CSF-dependent myeloid cell line (NFS-60) whose phosphorylation levels are modulated by the growth inhibitor 8-bromo-cAMP (8BrcAMP). Evidence is presented from both this approach and kinase activity measurements that the enhanced Erk-1 activity due to G-CSF action was not down-regulated by exogenous 8BrcAMP; however, the G-CSF-enhanced Raf-1 activity was suppressed by 8BrcAMP, suggesting that Erk-1 might not be downstream of p21^{ras} or Raf-1 in these cells. Furthermore we examined by two-dimensional SDS/PAGE the similarities and differences between the phosphoprotein patterns resulting from the addition of exogenous Erk-1 and Raf-1 to NFS-60 cytosolic extracts, which support the notion that these kinases might lie on different pathways in these cells.

EXPERIMENTAL

Reagents

RPMI, Dulbecco's modified Eagle's medium (DMEM), streptomycin and penicillin were purchased from ICN-Flow Laboratories (Sydney, NSW, Australia), foetal bovine serum (FBS) was from Commonwealth Serum Laboratories (Parkville, Vic., Australia). Other reagents were obtained as follows: recombinant human G-CSF was purchased from Amgen (Thousand Oaks, CA, U.S.A.), sodium salt of 8BrcAMP from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and [*methyl*- ^3H]thymidine (70–85 Ci/mmol) was from Amersham (Sydney, Australia).

Cells and cell culture

NFS-60 is a G-CSF/interleukin 3-dependent murine cell line and was obtained from Dr. J. Layton (Ludwig Institute for Cancer Research, Parkville, Vic., Australia). Cells were grown in DMEM containing 2 mM glutamine, 10% (v/v) FBS and 1 ng/ml G-CSF at 37 °C in 5% CO₂ in air and were subsequently washed and incubated in DMEM with FBS without growth factor for 14 h before use to render them quiescent.

DNA synthesis

DNA synthesis was measured as the incorporation of [*methyl*- ^3H]thymidine as previously described. Quiescent NFS-60 cells (10^5 in 24-well plates) were incubated in G-CSF (1 ng/ml) in the presence or absence of 8BrcAMP (1 mM) with [*methyl*- ^3H]thymidine (2.5 $\mu\text{Ci}/\text{ml}$) added at 0 h. At 14 h cells were harvested on glass filters with an Inotech harvester, and read on a Bertold Digital Autoradiograph to measure incorporated radioactivity.

Immunoprecipitation kinase assay

Cytosolic cell lysates containing membrane and cytoplasmic compartments were prepared as follows: 10^7 cells were lysed in

lysis buffer [10 mM Tris/HCl, pH 7.2, containing 1% (v/v) NP40, 0.5% deoxycholate, 70 i.u./ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 mM NaF, 1 mM EDTA, 0.1 mM Pefabloc and 200 μM sodium orthovanadate], centrifuged at 5000 *g* and the pellets discarded. Immunoprecipitations were performed by incubating 20 μg of appropriately treated cell lysate with 1 μg of anti-p44^{MAPK} (Erk-1) or anti-Raf-1 antibodies (Santa Cruz Biotechnology, CA, U.S.A.) overnight at 4 °C. A 10% (v/v) slurry (50 μl) of Protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) was added and the mixture was incubated for 30 min at 4 °C. The beads were pelleted and washed three times with lysis buffer. Pellets were incubated with 20 μg of myelin basic protein (MBP) for Erk kinase assays or 5 μg of glutathione S-transferase (GST)-MEK (kinase-dead; Upstate Biotechnology, Lake Placid, NY, U.S.A.) for 20 min at 30 °C in 50 μl of kinase assay buffer containing 20 mM Hepes, pH 7.4, 10 mM MgCl₂, 0.2 mM ATP, 0.2 mM NaF, 0.1 mM sodium orthovanadate and 10 μCi of [γ - ^{32}P]ATP. Reactions were stopped by the addition of SDS/PAGE sample buffer. Samples were boiled for 5 min and subjected to one-dimensional SDS/PAGE, stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), dried and exposed to X-ray film.

Ras assay

Quiescent NFS-60 cells were ^{32}P -labelled by incubation in phosphate- and serum-free DMEM (ICN-Flow Laboratories, Sydney, Australia) for 3 h in the presence of 1 mCi of [^{32}P]P_i (Dupont, Boston, MA, U.S.A.) per 10^7 cells. These were stimulated with 1 ng/ml G-CSF and/or 1 mM 8BrcAMP, which was stopped by the addition of ice-cold PBS followed by centrifugation and washing with ice-cold PBS. Pelleted cells were lysed in 10 mM Tris/HCl, pH 7.2, containing 1% (v/v) NP40, 0.5% deoxycholate, 70 i.u./ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 mM NaF, 1 mM EDTA, 0.1 mM Pefabloc and 200 μM orthovanadate. The sample was centrifuged at 5000 *g* for 5 min and the pellet discarded. Lysates were precleared by 5 min incubation with a 10% (w/v) slurry of BSA-treated activated charcoal, and subsequently incubated with Y13-259 anti-p21^{ras} rat monoclonal antibody for 4 h at 4 °C. This was followed by a 1 h incubation with rabbit anti-(rat immunoglobulin) antibodies and a 30 min incubation with a 10% (v/v) slurry of Protein A-Sepharose 4B. Pellets were washed three times with 10 mM Tris/HCl, pH 7.2, containing 1% (v/v) NP40, 0.5% deoxycholate, 70 i.u./ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 mM NaF, 1 mM EDTA, 0.1 mM Pefabloc and 200 μM orthovanadate, and three times in 50 mM Tris/HCl, pH 7.5, containing 20 mM MgCl₂ and 150 mM NaCl. The pellets were then boiled for 3 min in 750 mM KH₂PO₄ (pH 3.4) containing 5 mM EDTA, 2 mM GMP, 2 mM GDP and 2 mM GTP and subjected to TLC with 0.5 M LiCl in 1.0 M acetic acid. TLC plates were dried and subjected to phosphorimager analysis. Counts for TLC-resolved nucleotide spots were determined and the degree of stimulation was calculated within an experiment.

Radiolabelling of cells and cell lysis

Quiescent NFS-60 cells ($10^6/\text{ml}$ in 175 cm² flasks) were incubated in phosphate- and serum-free DMEM with [^{32}P]P_i (1 mCi per 10^7 cells) for 2 h. Cells were then treated with 1 ng/ml G-CSF. Cell stimulation was stopped by the addition of ice-cold PBS followed by centrifugation and washing. Pelleted cells were lysed in 10 mM Tris/HCl, pH 7.2, containing 1% (v/v) NP40, 0.5% deoxycholate, 70 i.u./ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 mM NaF, 1 mM EDTA, 0.1 mM Pefabloc and 200 μM orthovanadate.

The sample was centrifuged at 5000 *g* for 5 min and the pellet discarded. Protein concentration determinations were performed were the Bio-Rad Protein Assay.

Two-dimensional SDS/PAGE

Cell lysates (100 μ g of protein) were subjected to isoelectric focusing (IEF) at 1810 V for 15.5 h at 18 °C with pH 3–10 ampholytes (Bio-Rad) using the Bio-Rad Protean 2 electrophoresis system. The procedure was modified from that described by O'Farrell [33] by using isoelectric focusing tubes with an internal diameter of 3 mm. After isoelectric focusing, the gel tubes were extruded with doubly distilled H₂O and equilibrated in SDS sample buffer containing 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 2.3 % (w/v) SDS and 62.5 mM Tris/HCl, pH 6, for 2 h at room temperature. The second-dimension separation was performed by SDS/PAGE [12.5 % (w/v) gel of 0.75 mm thickness] at a constant current of 25 mA per gel and the electrophoresis chamber was maintained at 4 °C. One- and two-dimensional SDS/PAGE gels were stained overnight with 0.1 % Coomassie Blue in 25 % (v/v) methanol with 10 % (v/v) acetic acid, destained in 25 % (v/v) methanol with 10 % (v/v) acetic acid, dried and subjected to autoradiography. All results are representative of at least five separate experiments. Wide-range protein standards were used to determine molecular mass in the second dimension (Novex, San Diego, CA, U.S.A.). Isoelectric point (pI) values were determined by two-dimensional SDS/PAGE pH 4.5–8.5 standards (Bio-Rad) and the Carbamylate Calibration Kit (Pharmacia).

Quantitative determination of phosphorylation

All two-dimensional SDS/PAGE gels were of cytoplasmic lysates containing 100 μ g of protein. Gel autoradiographs were scanned with a Computerized Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the images analysed with Image Quant software (Molecular Dynamics).

Western blot analysis of two-dimensional SDS/PAGE gels

Gels for analysis by Western blotting were transferred to Hybond-C membranes (Amersham, Little Chalfont, Bucks., U.K.). Membranes were blocked in BSA and ovalbumin-containing PBS (BOP) buffer [3 % (w/v) BSA/1 % (w/v) ovalbumin in PBS], incubated with rabbit polyclonal anti-p44^{MAPK} (Erk-1) antibody (1 μ g/ml) for 2 h at room temperature in BOP buffer. Membranes were washed twice in Tris-buffered saline for 10 min each (TBS), once in TBS with 0.1 % (v/v) Tween-20 for 10 min, twice in TBS for 10 min each, incubated with conjugated pig anti-rabbit antibodies (1:10⁴; Amersham) and washed as previously described. Western blots were developed by the enhanced chemiluminescence system (Amersham).

Exogenous kinase assay

Either agarose-bound GST-Raf-1 (1 μ g) or GST-ERK-1 (1 μ g) (Upstate Biotechnology) was incubated with 20 μ g of cytosolic lysate of quiescent NFS-60 cells for 20 min at 37 °C in 20 μ l of kinase assay buffer containing 20 mM Hepes, pH 7.4, 10 mM MgCl₂, 0.2 mM ATP, 0.2 mM NaF, 0.1 mM sodium orthovanadate and 10 μ Ci of [γ -³²P]ATP. Reactions were stopped by the addition of SDS/PAGE sample buffer. Samples were boiled for 5 min and subjected to two-dimensional SDS/PAGE, stained with Coomassie Brilliant Blue R-250, dried and exposed to X-ray film.

RESULTS

8BrcAMP inhibits G-CSF-mediated DNA synthesis in NFS-60 cells

Increased intracellular cAMP resulting, for example, from the addition of 8BrcAMP, can suppress cell proliferation in a number of cell types, including myeloid cells [19–23]. In Table 1 we show that 8BrcAMP is a potent inhibitor of G-CSF-induced DNA synthesis in NFS-60 cells; a similar suppression of the increase in cell numbers induced by G-CSF was also observed (results not shown). We therefore decided to use this system to explore both known and unknown potential targets for cAMP action that lie downstream of the G-CSF receptor.

8BrcAMP does not inhibit Erk-1 activation in G-CSF-treated NFS-60 cells

It has been reported that in some growth factor-sensitive cell types Erk species are a target for the anti-proliferative action of elevated intracellular cAMP [24–29]. We show in Figure 1, using immunoprecipitation kinase assays, that Erk-1 was activated maximally at approx. 5–15 min after G-CSF addition, irrespective of 8BrcAMP pretreatment. The levels of immunoprecipitated Erk-1 were found to be equal in all samples as determined by Western blot analysis (Figure 1). Given the apparent insensitivity of Erk-1 activation to 8BrcAMP we sought to examine likely upstream members of the MAP kinase cascade, namely p21^{ras} and Raf-1 [35–37], for their activation state in cells treated with 8BrcAMP.

8BrcAMP abrogates p21^{ras} activation in G-CSF-treated NFS-60 cells

The GTP-binding activity of p21^{ras} was elevated 2.4- and 2.7-fold by treatment with G-CSF (1 ng/ml) at 5 and 10 min respectively (Figure 2A). Pretreatment of NFS-60 cells with 1 mM 8BrcAMP before stimulation with 1 ng/ml of G-CSF for 10 min resulted in blocking of this enhanced GTP-binding activity (Figure 2B).

8BrcAMP blocks G-CSF-mediated Raf-1 activation in NFS-60 cells

We monitored Raf-1 activity in immunoprecipitates of cytosolic extracts by using the same approach as for Erk-1, but with GST-MEK (kinase-dead) fusion protein as substrate. The MEK-kinase activity of Raf-1 from G-CSF-treated NFS-60 cells was almost completely abrogated by pretreatment with 8BrcAMP (Figure 3). Raf-1 levels in all immunoprecipitated reactions were equal as determined by Western blot analysis. These Raf-1 activity measurements suggest that Erk activation might occur

Table 1 Inhibition of DNA synthesis in NFS-60 cells treated with G-CSF and 8BrcAMP

Quiescent NFS-60 cells were untreated or treated with 8BrcAMP alone (1 mM), G-CSF alone (1 ng/ml) or G-CSF and 8BrcAMP in the presence of [*methy*³H]thymidine and the cells were harvested at 14 h (see the Experimental section). Inhibition of DNA synthesis was determined by comparing [*methy*³H]thymidine incorporation (in c.p.m.) with that induced by G-CSF alone. The results are from a typical experiment that was repeated four times. Values are the means $\pm$ S.E.M. for triplicate cultures.

Treatment	Mean thymidine incorporation (c.p.m.)
Untreated	272.00 $\pm$ 36.38
G-CSF alone	2028.33 $\pm$ 200.65
8BrcAMP alone	248.00 $\pm$ 9.54
G-CSF and 8BrcAMP	297.00 $\pm$ 56.00

Figure 1 Kinetics of activation of Erk-1 in NFS-60 cells treated with G-CSF and 8BrcAMP

Quiescent NFS-60 cells were treated with G-CSF (1 ng/ml) for the times indicated, either with or without 8BrcAMP (1 mM). The cells were lysed and Erk-1 was immunoprecipitated from the cytosolic fractions. Washed pellets were assayed for MBP kinase activity as outlined in the Experimental section. Coomassie Blue-stained MBP is shown as a control for loading, as is a Western blot of Erk-1 protein levels as a control for Erk-1 loading. Abbreviations: α-, anti-; i.p., immunoprecipitation.

Figure 2 G-CSF activation of p21^{ras} and its inhibition by 8BrcAMP

Quiescent NFS-60 cells (10^7) were labelled with 1 mCi of [32 P] P_i for 3 h. (A) Cells were treated with G-CSF (10 ng/ml) for the times indicated, lysed and incubated with (+) or without (-) Y13-259 anti-p21^{ras} rat monoclonal antibody, and p21^{ras} was measured as in the Experimental section. Activation of p21^{ras} is shown as a fold increase, as determined by phosphorimager analysis of the GTP/GDP ratios. (B) Cells were treated with (+) or without (-) G-CSF (1 ng/ml) for 10 min in the presence (+) or absence (-) of 8BrcAMP (1 mM), as indicated, and assayed as described above. Activation of p21^{ras} is shown as a fold increase in the GTP/GDP ratio as the mean $\pm$ S.E.M. for triplicate cultures from a representative of four experiments. Abbreviation: α-, anti-.

via a pathway other than the conventional one involving upstream Raf-1 phosphorylation of MEK in this system (see the Discussion section). Given the above findings, it was reasoned that an analysis of the effect of 8BrcAMP on the phosphorylation status of proteins in G-CSF-treated NFS-60 cells might provide information not only on the mode of action of this inhibitor but

Figure 3 G-CSF activation of Raf-1 and its inhibition by 8BrcAMP

Quiescent NFS-60 cells (10^7) were treated with G-CSF (1 ng/ml) for 15 min either with (+) or without (-) 8BrcAMP (1 mM). The cells were lysed, cytosolic extracts were prepared as described in the Experimental section, and Raf-1 was immunoprecipitated. Washed pellets were assayed for GST-MEK-kinase activity as outlined in the Experimental section. Coomassie Blue-stained GST-MEK is shown as a control for loading, as is the immunoglobulin heavy chain of anti-Raf-1 antibodies (IgHc). A Western blot of Raf-1 protein levels is shown as a control for Raf-1 loading. These results are typical of three experiments. Abbreviation: α-, anti-.

also on molecules lying upstream and/or downstream of Raf-1 and/or Erk species in these cells. Two-dimensional SDS/PAGE analysis of cytosolic lysates from 32 P-labelled cells was chosen for this purpose.

Effect of 8BrcAMP on the two-dimensional SDS/PAGE profile of 32 P-labelled proteins in G-CSF-treated NFS-60 cells

We have previously demonstrated that two-dimensional SDS/PAGE of 32 P-labelled cellular protein extracts has the potential to analyse the signal transduction pathways used by G-CSF action in these cells and to investigate the action of growth inhibitors [32a]. 32 P-labelled NFS-60 cells were subjected to four treatments *in vivo*, namely unstimulated, 8BrcAMP alone, G-CSF alone or G-CSF and 8BrcAMP together: 145 distinct spots were observed after the treatments detailed above at the 15 min time point (Table 2). This time was chosen as the point at which the greatest number of phosphorylation events were evident [32a]. Four spots not present in untreated quiescent cells (i.e. cells starved of G-CSF) became phosphorylated after the addition of 8BrcAMP alone (Table 2). G-CSF treatment of NFS-60 cells resulted in the modulation of the phosphorylation

Table 2 Effect of 8BrcAMP on the two-dimensional SDS/PAGE profile of ³²P-labelled proteins in G-CSF-treated NFS-60 cells

Quiescent NFS-60 cells were prelabelled with [³²P]P_i (1 mCi per 10⁷ cells) and were either unstimulated or treated before lysis with G-CSF (1 ng/ml) for 15 min with or without 8BrcAMP (1 mM, 30 min pretreatment). Cytosolic extracts were subjected to two-dimensional SDS/PAGE as described in the Experimental section, and to autoradiography. The radiolabelled spots were quantified by computerized densitometry and arbitrarily assigned a 'spot number'. Only those spots whose positions could be represented clearly and separately in Figure 4 have been included. The relative intensities were standardized in relation to that of an invariant phosphoprotein, no. 272, as follows: —, equal to background; +/—, 2-fold or less above background; +, 2–3-fold above background; ++, 3–5-fold above background; +++, 5–10-fold above background; +++, more than 10-fold above background.

Spot no.	Unstimulated		G-CSF		Spot no.	Unstimulated		G-CSF		Spot no.	Unstimulated		G-CSF		Spot no.	Unstimulated		G-CSF	
	-cAMP	+cAMP	-cAMP	+cAMP		-cAMP	+cAMP	-cAMP	+cAMP		-cAMP	+cAMP	-cAMP	+cAMP		-cAMP	+cAMP	-cAMP	+cAMP
1	-	+	-	+	49	-	+	-	++	106	+/-	+/-	+/-	+	198	-	-	+++	+++
2	-	-	+	+	50	+	+	++	++	107	-	-	+	+	199	-	-	+	+
3	-	-	+	+	51	+	+	++	++	108	-	-	+	+	200	-	-	++	++
4	-	+/-	+	++	52	+	+	++	++	109	-	-	+	+	202	-	-	-	++
5	-	+/-	+	++	53	-	-	+	+	110	+/-	+/-	+	+	203	-	-	+/-	+/-
6	-	-	-	+	55	-	-	+	+	111	-	-	+/-	+/-	204	-	-	+/-	+/-
7	-	-	-	+	56	-	-	-	+	115	-	-	++	++	205	-	-	+/-	+/-
8	-	-	++	+++	57	-	-	+	+	116	-	-	+	+	213	-	-	+/-	+/-
9	-	-	+/-	++	58	-	-	+	+	117	-	-	+	+	215	-	-	+	+
10	-	-	+	+	59	-	-	+	+	118	-	-	+	+	218	+/-	+/-	+	+
11	-	-	-	+	60	-	-	+	+	119	-	-	+	+	221	-	-	+	+
12	-	-	-	+	63	-	+++	-	-	123	-	-	-	++	222	+/-	-	+/-	+
13	-	-	+	+	65	-	-	-	+/-	124	-	-	+	+	225	-	-	+	+
14	-	+	+	++	69	-	-	-	+	126	+	-	++	++	226	+/-	-	++	++
15	+	+	+	+++	70	-	-	+/-	+	127	+	+	++	++	227	+	+	+/-	+/-
16	+	+	+	+++	73	-	-	+	+	130	-	-	+	+	228	-	-	+	+
17	+	+	+	++	74	-	+	+	++	131	+	+	+	-	229	-	-	-	+
21	+	+	++	++	78	-	-	++	-	134	-	-	+	+	230	-	-	-	+
22	-	-	-	++	80	-	-	+	+	156	-	-	-	++	240	-	-	+	+
23	-	-	++	++	81	-	-	+	+/-	157	+/-	+/-	+	++	241	-	-	+/-	+
24	-	-	++	++	82	-	-	-	+	159	-	-	++	-	242	++	++	+++	+++
25	-	-	+	+	83	-	-	+	+	161	-	-	-	++	245	-	-	-	+
26	-	-	++	++	84	-	-	+	+	166	-	-	+	++	246	-	-	-	+
27	-	-	++	++	85	-	-	++	++	167	-	-	+	++	248	-	-	-	+
30	-	-	+++	+++	86	-	-	++	++	178	+	+	++	++	249	-	-	-	+
31	-	-	+++	+++	88	-	-	+	+	181	+/-	+/-	+	+	250	-	-	++	++
32	-	-	+++	+++	89	-	-	++	++	182	+/-	+/-	+	+	251	-	-	++	++
33	-	-	-	++	90	-	-	++	++	183	+/-	+/-	+	+	252	+/-	+/-	++	++
35	-	-	+	+	91	+/-	+	++	+	185	-	-	+	+	253	+/-	-	+	-
36	+	-	++	+	92	+/-	+	++	+	186	-	-	+	+	254	-	-	+/-	+
39	-	-	+	+	93	+/-	-	++	+	187	-	-	-	+	255	-	-	+	++
40	-	-	+	+	99	+/-	+	+	+	188	-	-	+	+	256	++	++	+++	+++
41	-	-	+	+	100	-	-	+/-	+	189	-	-	-	+	258	+	+	+++	+++
42	-	-	+	+	101	+/-	+	+	+	190	-	-	-	+	262	-	+	++	++
43	-	-	+	+	102	+/-	+	+	+	192	+	+	+	+	272	+++	+++	+++	+++
45	-	-	+	+	104	+/-	-	+	+	193	-	-	-	+					
48	-	-	+	-	105	+/-	+/-	+/-	++	197	-	-	++	++

Figure 4 Two-dimensional SDS/PAGE map of phosphorylated proteins derived from NFS-60 cells

Quiescent NFS-60 cells were pre-labelled with [32 P]P_i (1 mCi per 10⁷ cells) treated with G-CSF (1 ng/ml) alone, with 8BrcAMP (1 mM) alone or with both together. The cells were extracted and the cytosolic extracts were subjected to two-dimensional SDS/PAGE as described in the Experimental section. Proteins that were significantly phosphorylated in each of these extracts were arbitrarily assigned a number based on their molecular mass (molecular weight) and isoelectric point values and represented in a combined diagram. The resulting map was generated with a Computerized Laser Densitometer. The map does not represent the intensities of the different spots.

state of 97 spots (96 phosphorylated and one dephosphorylated by G-CSF treatment). Of the G-CSF-modulated spots, 82 were unaffected by pretreatment with 8BrcAMP, and the remaining 15 spots were. In addition to these spots, 44 phosphoproteins were induced only by treatment with the combination of 8BrcAMP and G-CSF (Table 2). Any of the 59 G-CSF-modulated spots affected by 8BrcAMP treatment could represent downstream targets for the growth-inhibitory action of 8BrcAMP.

For the purposes of identification, each 32 P-labelled spot was given an arbitrary number. All spots found that were modulated by treatment with G-CSF alone, 8BrcAMP alone or G-CSF and 8BrcAMP together are presented in a composite diagram in Figure 4. This diagram simply represents the apparent molecular masses and pI values with no reference to the intensity of each spot. In Table 2 the actual relative intensities of the spots are represented by comparison with the intensity of an invariant 'land-mark' spot (no. 272).

Figure 5 provides a few examples of some of these various patterns. The phosphorylation of spots no. 8 (Figure 5A), 31 (Figure 5B), 126 (Figure 5C), and 197, 198 and 242 (Figure 5E) is enhanced by G-CSF treatment and this increase is unaffected by 8BrcAMP pretreatment. In contrast, the increased phosphate incorporation into spots no. 26 and 27 (Figure 5A) and 256 (Figure 5F) is abrogated by 8BrcAMP pretreatment. These spots seem to be examples of downstream targets for the growth-inhibitory effects of 8BrcAMP as described above. Spots no. 124 and 192 (Figures 5C and 5D respectively) are phosphorylated in response to 8BrcAMP independently of G-CSF. The changes in the phosphorylation states of these few examples are in marked contrast with the invariance of the standard protein spot no. 272 (Figure 5G) and spot no. 258 (Figures 4 and 5F). From our

Figure 5 Phosphorylation of cytosolic proteins induced by G-CSF is differentially affected by pretreatment with 8BrcAMP

(A-G) Quiescent NFS-60 cells were pre-labelled with [32 P]P_i (1 mCi per 10⁷ cells) and were treated with (+) or without (-) G-CSF for 15 min, after a 30 min pretreatment in the presence (+) or absence (-) of 8BrcAMP (1 mM). Cytosolic extracts were subjected to two-dimensional SDS/PAGE as described in the Experimental section, followed by autoradiography. The arrows indicate representative phosphoproteins discussed in the text. (H) Cytosolic extracts were subjected to two-dimensional SDS/PAGE and transferred to nitrocellulose as described above. Membranes were autoradiographed and then subjected to Western blot analysis using Erk-1-reactive antibodies. Both the autoradiographs and the Western blot are shown. Abbreviations: #, no.; α-, anti-.

observations presented above on the regulation of Erk activity, it might be predicted that the phosphorylation status of the Erk species would be altered by G-CSF-treatment and that this might not be altered in the presence of 8BrcAMP. As shown in Figure 5(H), we identified spots no. 109, 111, 115 and 118 (Figure 4), which increased in intensity on G-CSF addition (Figure 5H and Table 2), as corresponding to Erk-1 by Western blotting (Figure 5H). Pretreatment of cells with 8BrcAMP did not diminish the degree of 32 P incorporation in spots no. 109, 111, 115 and 118 (Figure 5H and Table 2). Thus the two-dimensional SDS/PAGE findings on the Erk system, as an example of a characterized protein, are consistent with the kinase activity results above (Figure 1).

Table 3 Identification of downstream targets/substrates in NFS-60 cell lysates treated with exogenous Erk-1 or Raf-1

Quiescent NFS-60 cell lysate was incubated with either agarose alone (untreated) or agarose-bound activated GST-Erk-1 or GST-Raf-1 in the presence of [γ - 32 P]ATP. Labelled cytosolic extracts were subjected to two-dimensional SDS/PAGE as described in the Experimental section, and to autoradiography. The radiolabelled spots were quantified by computerized densitometry and were arbitrarily assigned a 'spot number' on the basis of electrophoretic mobility due to molecular mass and isoelectric point, as described for labelling *in vivo* (see Figure 4). The level of phosphorylation of each spot was standardized in relation to that of an invariant phosphoprotein, no. 272, as follows: —, equal to background; +/—, 2-fold or less above background; +, 2–3-fold above background; ++, 3–5-fold above background; + + +, 5–10-fold above background; + + + +, more than 10-fold above background.

Spot no.	Kinase treatment <i>in vitro</i> ...	Phosphorylation intensity		
		Untreated	GST-Erk-1	GST-Raf-1
36		—	—	+
85		—	+ + + +	+ + + +
102		—	+ + + +	+ + +
192		+ +	+	+ + + +
197		+ / —	+	—
213		+ + +	—	+ / —
215		+ + +	—	+ / —
272		+ + + +	+ + + +	+ + + +

Identification of Raf-1 and Erk-1 substrates in NFS-60 cell lysates

We next considered the possibility that two-dimensional SDS/PAGE might be a useful tool to test the concept of Raf-1-independent activation of Erk species by examining the phosphorylation pattern(s) resulting from the addition of exogenous Erk-1 and Raf-1 to NFS-60 cell extracts. The identification of distinct phosphoproteins induced in this way by each kinase would be suggestive of distinct kinase cascades, whereas the appearance of common phosphoproteins might argue for the same or convergent pathways.

Cytosolic extracts of quiescent NFS-60 cells were incubated with either GST-Erk-1 or GST-Raf-1 in the presence of [γ - 32 P]ATP. Extracts kinase-labelled *in vitro* were subjected to two-dimensional SDS/PAGE and autoradiography (Table 3). The electrophoretic mobility of each phosphoprotein was compared with those phosphorylated *in vivo* (Tables 2 and 3, and Figures 4 and 5). We have included in Table 3 only those spots that were readily detectable on a short exposure and were also found *in vivo*, because the significance of substrates/targets not found *in vivo* is unknown. Also not included are those constitutive spots whose intensities were not altered by exogenous kinase addition. Representative examples of some of these spots are depicted in Figure 6. Given these criteria, the spots generated by exogenous kinase were observed to fall into three categories: modulated by GST-Erk-1 alone (no. 197; Table 3 and Figure 6C), by GST-Raf-1 alone (no. 192; Table 3 and Figure 6B), or by both kinases, either up-regulated (nos. 85, 102; Table 3 and Figure 6A) or down-regulated (nos. 213, 215; Table 3 and Figure 6D). The intensities of phosphorylation of the various spots were once again rated in comparison with that of the standard spot no. 272 (see above), whose intensity was again unaffected, this time by incubation in the absence or presence of either exogenous kinase (Table 3; Figure 6E). The presence of this last group of common phosphoproteins suggests that both Erk-1 and Raf-1 might act directly or indirectly on some common substrate/target(s) that are upstream of spots no. 85 and 102, or on these proteins themselves; the disappearance of spots no. 213 and 215 suggests that both kinases might be involved in one or more signalling pathways that includes a common phosphatase.

Figure 6 Phosphorylation patterns after treatment of NFS-60 cell lysates with exogenous Erk-1 or Raf-1

Quiescent NFS-60 cell lysates were incubated with either agarose alone or agarose-bound GST-Erk-1 or GST-Raf-1 for 20 min at 37 °C with [γ - 32 P]ATP. Labelled extracts were subjected to two-dimensional SDS/PAGE as described in the Experimental section, followed by autoradiography. The arrows indicate phosphoproteins discussed in the text and in Table 3. Abbreviation: #, no.

As regards the sensitivity or otherwise to 8BrcAMP *in vivo*, it might be expected that spots appearing only after exogenous Erk-1 treatment might be unaltered by 8BrcAMP treatment; this is so (no. 197, Tables 2 and 3). In contrast, spots that appear *in vitro* only after treatment with exogenous Raf-1 might be expected to be altered by 8BrcAMP *in vivo*; this is so with spots no. 36 and 192 (Tables 2 and 3). Spots that are affected by both exogenous Erk-1 and Raf-1 *in vitro* (nos. 85, 102, 213 and 215; Table 3) are not regulated by treatment with 8BrcAMP *in vivo* (Table 2), suggesting that cAMP-independent Erk-1 is capable of compensating for the inactivation of Raf-1 by cAMP with respect to the regulation of the phosphorylation state of common targets.

Our current understanding of the MAPK cascade suggests that Erk-1 might be phosphorylated *in vitro* by exogenous Raf-1 [8,9]. However, spots corresponding to Erk-1 were observed to be phosphorylated in kinase experiments *in vitro* irrespective of the addition or type of kinase added (results not shown). Cellular decompartmentalization associated with the kinase assay *in vitro* might result in the breakdown of normally operating regulatory mechanisms. It is therefore impossible to comment on the ability of GST-Raf-1 to mediate the phosphorylation of Erk species in this system.

DISCUSSION

The immunoprecipitation kinase assay results showing that 8BrcAMP did not affect Erk activation in G-CSF-treated NFS-60 cells (Figure 1) suggests that, in contrast with a number of cellular systems [15,25,26,29], Erk activity might not be changed by elevated intracellular cAMP. As assayed by two-dimensional

SDS/PAGE, the unchanged phosphorylation pattern of the spots assigned to Erk-1 in the presence of 8BrcAMP (Figure 5H) is consistent with this observation. In systems where 8BrcAMP has been demonstrated to decrease Erk activity, the decline in activity seems to be due to a decrease in Raf-1 activity acting upstream of Erk. In the G-CSF-treated NFS-60 cells, Raf-1 activity was indeed decreased by exogenous 8BrcAMP (Figure 3) as was that of p21^{ras} (Figure 2). We are not aware of any other reports showing that such enhanced p21^{ras} activity can be inhibited by elevated cAMP. These findings are suggestive of a Raf-1 dependent pathway with a minor contribution to the activation of Erk in this system, although we cannot exclude the possible contribution of any residual Raf-1 activity remaining after 8BrcAMP treatment. The ability of 8BrcAMP to abrogate p21^{ras} activation by G-CSF in NFS-60 cells suggests that elevated intracellular cAMP levels might act, presumably via protein kinase A, at a point upstream of p21^{ras} and Raf-1. Alternatively, p21^{ras} might not be upstream of Raf-1 and is affected independently. In other words, the typical p21^{ras}/Raf-1/MEK/MAPK signalling pathway [36,37] might not be that significant in the response of these cells to G-CSF. Other studies have also demonstrated that this pathway might not be universally operative or linear; for example, MEK activation has been shown to occur independently of Raf-1 activation in epidermal growth factor-treated COS-7 and Swiss 3T3 cells [15], and the expression of a dominant-negative p21^{ras} mutant in macrophages indicated p21^{ras}-independent and -dependent activation of Raf-1 and MAPK [14]. Additional experiments, for example with dominant-negative Raf-1 or p21^{ras} mutants in myeloid cells, are needed to substantiate the hypothesis.

Unlike Erk activity, p21^{ras} and Raf-1 activity was correlated with subsequent DNA synthesis in the presence of 8BrcAMP. This finding is consistent with a role for p21^{ras} and Raf-1 in the pathway(s) leading to DNA synthesis in response to G-CSF. However, the contrasting lack of correlation with the level of Erk activity could mean that Erk species are not directly implicated in the proliferative response of NFS-60 cells to G-CSF, as has been suggested by the weak p42^{MAPK} activation in cells transfected with a truncated G-CSF receptor that is incapable of transmitting a mitogenic signal [6]; however, it could be argued that the site(s) of the inhibitory action of elevated cAMP might be downstream of the Erk species.

The evidence obtained above for a dissociation of Raf-1 activation from Erk activation prompted us to employ two-dimensional SDS/PAGE to compare the phosphoprotein profiles emanating from the addition of exogenous Erk-1 and Raf-1 to NFS-60 cell lysates. Both unique and common ³²P-labelled spots were observed in response to each kinase, indicating that distinct and shared pathways might exist *in vivo*. Interestingly, the 8BrcAMP-dependence or otherwise of the intensities of these spots *in vivo* was consistent with their phosphorylation *in vitro* by respective exogenous kinases (Table 3), providing some validation for this strategy. Characterization of these spots identified by two-dimensional SDS/PAGE will be critical in defining the Raf-1- and Erk-dependent pathways involved in the action of G-CSF.

We have previously used two-dimensional SDS/PAGE to compare the phosphoprotein labelling in NFS-60 cells treated with G-CSF, interleukin 3 and CSF-1 (M-CSF), where we found both shared and cytokine-specific phosphorylation patterns [32a]. Using the same approach we have explored the ³²P-labelling of phosphoproteins in NFS-60 cells treated with G-CSF in the absence or presence of the proliferation inhibitor 8BrcAMP, and also with 8BrcAMP alone. A number of spots remained unchanged irrespective of the treatment and some were phosphory-

lated by 8BrcAMP treatment alone, whereas 8BrcAMP affected the phosphorylation levels of certain G-CSF-modulated spots but not of many others. Belonging to the latter class of spots were those that could be attributed to Erk-1 by Western blotting. The proteins whose phosphorylation state was altered by 8BrcAMP treatment of G-CSF-treated cells remain to be characterized; such characterization might help to define the kinase cascade(s) that can be regulated by enhanced intracellular cAMP to inhibit proliferation, presumably as a result of the activation of protein kinase A.

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