The EMBO Journal vol.5 no.11 pp.2889–2898, 1986

Early phosphorylation events following the treatment of Swiss 3T3 cells with bombesin and the mammalian bombesin-related peptide, gastrin-releasing peptide

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Communicated by R.Dulbecco

Bombesin and the related mammalian peptides, such as gastrin-releasing peptide (GRP), are potent mitogens for some fibroblast cell lines. Here we have examined the bombesin- and GRP-mediated changes in the phosphorylation of proteins in Swiss 3T3 cells and compared these to the events observed after platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and tumor promoter treatment. In agreement with previous reports, bombesin, GRP and PDGF, but not EGF, increased the activity of protein kinase C. This was assayed by an inhibition of [^{125}I]EGF binding, stimulation in phosphorylation of pp60$^{c\text{-}src}$ on serine 12 and stimulation in phosphorylation of a group of 80 kd proteins. The different phosphorylated forms of the 80 kd proteins were examined by tryptic peptide mapping and shown to contain multiple phosphorylation sites. An investigation of the tyrosine phosphorylation events following mitogen treatment revealed a significant difference between PDGF and the bombesin peptides. PDGF treatment caused a marked increase in total cellular phosphotyrosine levels, and tyrosine phosphorylation both of known substrates and its own receptor. In contrast, bombesin and GRP treatments resulted in only a weak or undetectable increase in tyrosine phosphorylation of total cellular protein or known substrates. In this respect bombesin and GRP were more similar to EGF. The fact that the bombesin peptides do not induce a phosphorylation response identical with either PDGF or EGF suggests that there is not a single common signal pathway which is activated by all these mitogens.

Key words: bombesin/GRP/growth factors/protein-tyrosine kinase/tumor promoters

Introduction

The binding of a number of polypeptide growth factors, such as platelet-derived growth factor (PDGF), to their specific cell-surface receptors causes an increase in protein-tyrosine kinase activity, which is integral to the receptor. Following ligand binding, the receptor itself is autophosphorylated and, in addition, a number of other substrate proteins are phosphorylated on tyrosine (reviewed in Cooper and Hunter, 1983; Hunter and Cooper, 1985). Treatment of the cells with these growth factors can also result in the stimulation of serine and threonine phosphorylation of target proteins. It has been proposed that many of these latter phosphorylation events are due to the activation of a cellular serine/threonine-specific protein kinase termed protein kinase C. This would occur via the growth factor stimulation of phosphatidylinositol (PI) breakdown, which generates a transient intermediate, diacylglycerol (DAG), that binds to and activates protein kinase C (reviewed in Nishizuka, 1984; Ashendel, 1985). Protein kinase C also acts as a major cellular receptor for tumor promoters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and phorbol dibutyrate (PDBu), which can substitute for DAG in the activation of the enzyme. Thus, activation of protein kinase C is likely to play an essential role in the mitogenic stimulation elicited by tumor promoters (reviewed in Nishizuka, 1984; Ashendel, 1985).

Bombesin is a tetradecapeptide isolated from the skin of *Bombina bombina* (Anastasi *et al.*, 1971). Several peptides structurally related to bombesin have been isolated from mammalian tissue and they are characterized by having a pyroglutamyl N terminus and a highly homologous C-terminal octapeptide. The best characterized is gastrin-releasing peptide (GRP; McDonald *et al.*, 1979), which competes with bombesin for binding to high-affinity cell surface receptors on target cells (Westendorf and Schonbrunn, 1983; Zachary and Rozengurt, 1985) and elicits identical responses when injected into experimental animals (Brown *et al.*, 1980). The bombesin-related peptides have a wide range of physiological effects including the stimulation of hormone release *in vivo* and *in vitro* (e.g. Rivier *et al.*, 1978; Westendorf and Schonbrunn, 1982; Swope and Schonbrunn, 1984) and stimulation of cell division in cultured fibroblasts (Rozengurt and Sinnett-Smith, 1983) and human bronchial epithelial cells (Willey *et al.*, 1984).

Recently it has been suggested that bombesin-related peptides are involved in the autocrine stimulation of human small-cell lung carcinomas as these cells respond mitogenically to and express high-affinity receptors for bombesin-related peptides (Weber *et al.*, 1985; Moody *et al.*, 1985), secrete large quantities of such peptides (Moody *et al.*, 1981; Erisman *et al.*, 1982), and show diminished proliferation in the presence of monoclonal antibodies that bind specifically to the C terminus of bombesin and the related peptides (Cuttitta *et al.*, 1985). In order to understand better the mechanism by which bombesin-related peptides may be involved in tumor growth we have examined some early bombesin-mediated responses in Swiss 3T3 cells.

Bombesin and the bombesin-related peptides are potent mitogens for Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983). This mitogenic stimulation is exerted via their binding to a single class of about 100 000–300 000 high-affinity cell surface receptors per cell (Zachary and Rozengurt, 1985; Brown and Laurie, 1986). Binding is accompanied by the breakdown of phosphoinositides (Brown *et al.*, 1984) and the subsequent activation of protein kinase C (Zachary *et al.*, 1986) as measured by increased phosphorylation of a cellular protein of 80 000 daltons, termed 80K (Rozengurt *et al.*, 1983; Blackshear *et al.*, 1985, 1986; Rodriguez-Pena and Rozengurt, 1986). We have confirmed these observations using a more direct assay for the activation of protein kinase C, the phosphorylation of pp60$^{c\text{-}src}$ on serine 12 (Gould *et al.*, 1985). We have also examined the possibility that, as with other growth factors, the binding of

Table I. Effect of mitogen treatment on incorporation of [^{32}P]orthophosphate, inhibition of EGF binding, and [^{3}H]thymidine incorporation in Swiss 3T3 cells

	Control	Bombesin	GRP	PDGF	EGF	TPA	PDBu
Whole cell PAA[a]							
% Phosphotyrosine	0.031	0.039	0.044	0.334	0.038	0.044	0.039
% Phosphothreonine	8.75	8.52	8.08	9.26	8.71	9.26	9.82
% Phosphoserine	91.22	91.44	91.88	90.41	91.25	90.70	90.14
[^{3}H]Thymidine incorporation (c.p.m. × 10^3)[b]	4.1 ± 0.1	127 ± 17	192 ± 30	611 ± 37	190 ± 37	202 ± 14	120 ± 14
EGF binding (c.p.m.)[c]	1446 ± 91	459 ± 2	ND	ND	ND	99 ± 10	95 ± 5

In all cases quiescent Swiss 3T3 cells were treated with the following concentrations of mitogens: 1 nM PDGF, 10 nM bombesin, 10 nM GRP, 5 nM EGF, 50 nM TPA, 300 nM PDBu.

[a]Cells were labeled for 18 h in phosphate-free DMEM containing 5% (v/v) DMEM and 2 mCi/ml ^{32}P-orthophosphate, and were then treated for 15 min with mitogens prior to lysis. The levels of individual phosphoamino acids are expressed as a percentage of the total phosphoamino acid content.

[b]Dishes of quiescent cells were incubated for a further 24 h in DMEM alone. Mitogens were added with 1 μg/ml insulin for a further 24 h and the cells were labeled with [^{3}H]thymidine for the final 6 h as described in Materials and methods. 10% FBS treatment resulted in a [^{3}H]thymidine incorporation value of 493 ± 47.5 × 10^3 c.p.m. Control values are those for cells cultured in the presence of insulin alone. Figures given are the mean values for three replicate samples.

[c]Cells were incubated for 18 h in phosphate-free DMEM containing 5% (v/v) DMEM and then treated for 60 min with mitogens and [^{125}I]EGF, and then cell-associated [^{125}I]EGF was measured as described in Materials and methods. Figures given are the mean value for three replicate samples.

bombesin and GRP to target cells is accompanied by an increased protein-tyrosine kinase activity in addition to the elevation of protein kinase C activity.

Results

Changes in cellular phosphotyrosine levels in vivo

To examine the phosphorylation events following bombesin treatment of intact cells, we used a bombesin-responsive clone of Swiss 3T3 cells expressing >100 000 bombesin receptors per cell (Zachary and Rozengurt, 1985; Brown and Laurie, 1986). These cells also have high-affinity receptors for PDGF and epidermal growth factor (EGF) and respond mitogenically to these factors (Collins and Rozengurt, 1984; Brown *et al.*, 1984; Zachary *et al.*, 1986; Table I). Cells were cultured for 7 days until they were quiescent and dishes were then treated in parallel in a number of experiments described below. This series of experiments was performed on three separate occasions with similar results.

Initially we measured whether bombesin or GRP binding to Swiss 3T3 cells was accompanied by an elevation of cellular protein phosphotyrosine levels similar to that previously described for PDGF treatment of these cells (Cooper *et al.*, 1982). For this purpose, dishes of quiescent cells were either labeled for 18 h with [^{32}P]orthophosphate, treated with mitogens for 15 min and then prepared for whole-cell phosphoamino acid analysis, or cultured for a further 24 h before addition of mitogens and 1 μg/ml of insulin to measure stimulation of DNA synthesis. PDGF was a potent mitogen for these cells. In agreement with previously published results (Collins and Rozengurt, 1982; Rozengurt and Sinnett-Smith, 1983; Corps *et al.*, 1985), we found that treatment of Swiss 3T3 cells with either bombesin, GRP or the tumor promoters, TPA and PDBu, in the presence of insulin resulted in a significant stimulation of [^{3}H]thymidine incorporation (25–40% of the maximal serum-stimulated value; Table I and legend). EGF was a moderately good mitogen for Swiss 3T3 cells stimulating [^{3}H]thymidine incorporation to approximately the same level as bombesin, GRP and the tumor promoters, but always less than serum or PDGF.

In resting cells very little (~0.03%) of the phosphate incorporated into cellular proteins was present on tyrosine residues. Treatment of the cells with PDGF sufficient to saturate the cell surface receptors for 15 min prior to lysis resulted in a 10-fold increase in the percentage of phosphate present in protein as phosphotyrosine. By contrast, treatment with saturating doses of bombesin, GRP or either of the two tumor promoters resulted in only a marginal increase in the level of cellular phosphotyrosine (Table I). The increment with the bombesin peptides was similar to that with the tumor promoters and could be a consequence of activation of protein kinase C (see below). In agreement with published results, EGF treatment of Swiss 3T3 cells had little effect on the levels of cellular phosphotyrosine (Cooper *et al.*, 1982), despite the fact that its receptor has ligand-stimulated protein-tyrosine kinase activity.

Phosphorylation of cellular protein-tyrosine kinase substrates

The effect of bombesin and GRP on the phosphorylation of cellular proteins was examined in more detail by resolving phosphoproteins from mitogen-treated cells in two-dimensional gels. We were particularly interested in the phosphorylation of three previously characterized proteins, p36, p42 and 80K. p36 was initially identified as a major phosphoprotein in chicken cells transformed with Rous sarcoma virus, which encodes the protein-tyrosine kinase, pp60$^{v\text{-}src}$ (Radke and Martin, 1979). An increased tyrosine phosphorylation of p36 in fibroblasts treated with PDGF has been previously observed (Cooper *et al.*, 1982; Ralston and Bishop, 1985; Isacke *et al.*, 1986). p42 is a low abundance protein which is present in mitogen-stimulated cells as a pair of related phosphoproteins (pp42A and pp42B) containing phosphotyrosine, as well as phosphoserine and in some cases phosphothreonine (Cooper *et al.*, 1984; Cooper and Hunter, 1985). The 80-kd phosphoproteins, which migrate at the acidic end of isoelectric focusing gels, have been identified as prominent phosphorylation substrates in cells treated with both tumor promoters and growth factors (Rozengurt *et al.*, 1983; Rodriguez-Pena and Rozengurt, 1985; Bishop *et al.*, 1985; Blackshear *et al.*, 1985, 1986). It has been proposed that the phosphorylation of these proteins, referred to as 80K, is mediated by protein kinase C.

Dishes of cells were labeled overnight with [^{32}P]orthophosphate in parallel with those assayed for whole-cell phosphoamino acid levels (see Table I) and lysed 15 min after the addition of mitogens. The phosphoproteins were separated by two-dimensional gel electrophoresis and the gels were then treated with alkali to hydrolyse the majority of serine-bound phosphate groups. PDGF stimulated the alkali-stable phosphorylation of a number

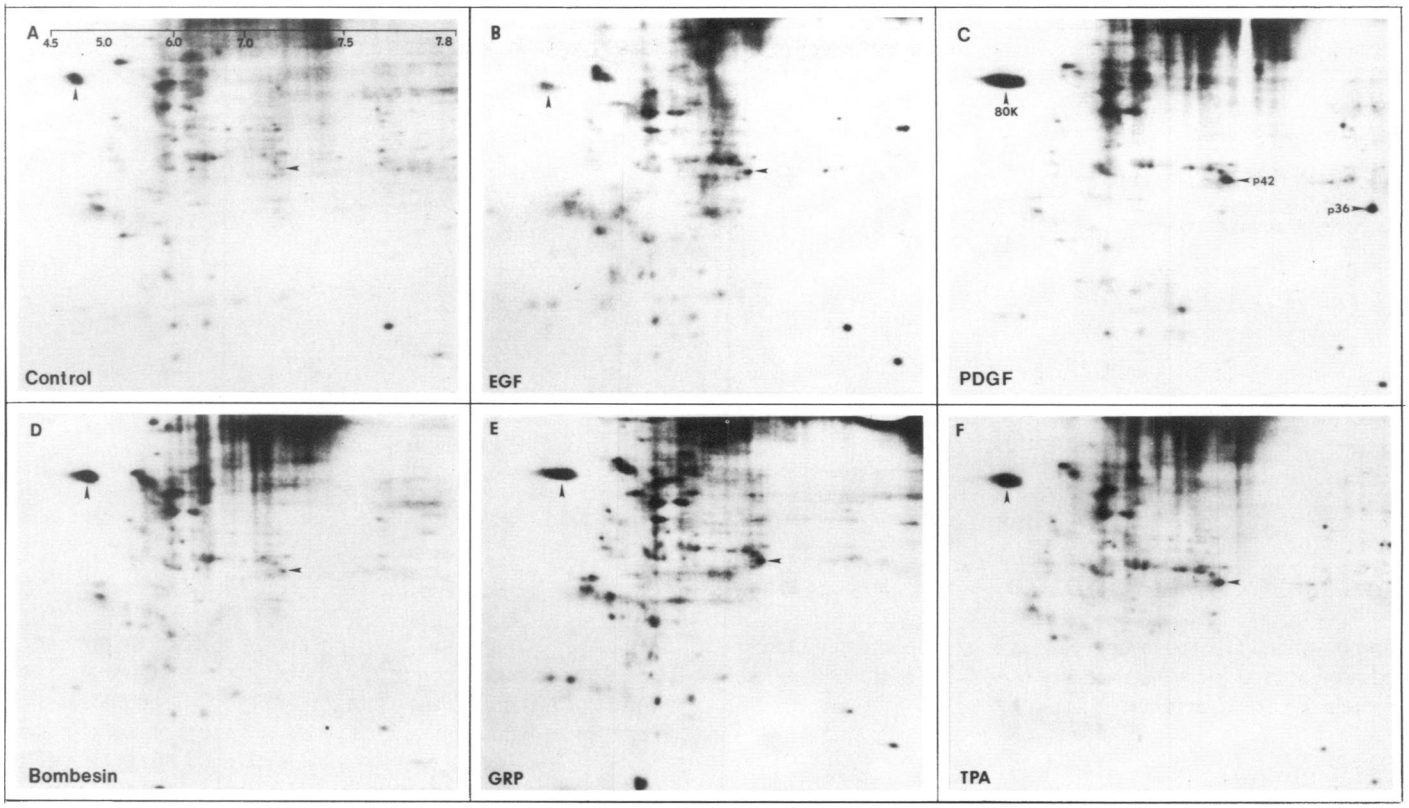

Fig. 1. Two-dimensional gel electrophoresis of ^{32}P-labeled proteins from mitogen-treated Swiss 3T3 cells. 35 mm dishes of quiescent Swiss 3T3 cells were labeled for 18 h with [^{32}P]orthophosphate as described in Materials and methods and mitogens were added directly to the labeling medium for 15 min prior to lysis. (**A**) No addition, (**B**) bombesin, 10 nM; (**C**) PDGF, 1 nM; (**D**) TPA, 50 nM; (**E**) EGF, 5 nM; (**F**) GRP, 10 nM. One-tenth of each sample was analysed on two-dimensional gels as described in Materials and methods. After fixation, gels were treated with alkali. Basic proteins are to the left of the gel; an approximate pH scale for the isoelectric focusing dimension is given in panel A. Arrowheads indicate the positions of pp42 (pointing left), pp36 (pointing right), and 80K (pointing upwards). Exposure times with an intensifying screen were 2 days. This experiment was performed in parallel with those described in Table I.

of proteins including p36, p42 and 80K (Figure 1). Consistent with previously published results, TPA treatment did not result in any observable alkali-stable p36 phosphorylation but did cause a dramatic increase in p42 phosphorylation and 80K phosphorylation (Figure 1; see next section). PDBu treatment of cells induced a series of phosphorylation events identical to that observed with TPA (data not shown). Two-dimensional resolution of the phosphoproteins extracted from bombesin- or GRP-treated cells gave a similar pattern to that from tumor promoter-treated cells, but the stimulation of 80K phosphorylation by bombesin or GRP was less than that observed with the tumor promoters or PDGF, while the stimulation of p42 phosphorylation was detectable but weak (Figure 1). The increase in alkali-stable phosphorylation of p42 in tumor promoter and bombesin- or GRP-treated cells may explain in part the small (but reproducible) increase in cellular phosphotyrosine levels in these cells (Table I). EGF treatment resulted in an increase in p42 but not p36 phosphorylation. Furthermore, no increase in 80K phosphorylation was observed suggesting that EGF treatment does not activate protein kinase C in these cells.

Phosphorylation of 80K in mitogen-treated cells

To obtain a quantitative estimate of the extent of 80K phosphorylation under various conditions, non-alkali treated gels were examined (Figure 2). It can be seen that 80K from mitogen-treated cells contained more acidic forms than that from untreated cells and that the most acidic of these tended to migrate more slowly than the most basic in the second dimension (Figure 2b). Up to seven discrete segments could be resolved in the 80K region.

To determine the number of phosphorylation sites in 80K and whether new sites were used upon mitogen stimulation, the whole group of ^{32}P-labeled 80K proteins was excised from untreated two-dimensional gels and subjected to phosphoamino acid analysis and two-dimensional tryptic peptide mapping (Figure 3). In quiescent cells 80K contained phosphoserine and phosphothreonine in a ratio of ~4:1. The mitogen treatments tested resulted in an increased serine phosphorylation of 80K with little change in threonine phosphorylation and no detectable tyrosine phosphorylation (Figure 3, insets). The presence of phosphothreonine may explain the alkali-stability of 80K. Tryptic peptide maps showed that 80K from untreated cells contained multiple phosphorylation sites which gave rise to 10 major tryptic phosphopeptides. All the mitogen treatments stimulated the phosphorylation of 80K at these same sites and at novel sites resulting in the appearance of phosphopeptides 1, 3 and 5 (Figure 3, see schematic diagram in Figure 4). The mitogen-stimulated appearance of the minor phosphopeptides, which migrated between peptides 7 and 11 and between peptides 8 and 10, was not reproducible (see Figure 4).

To examine the difference between the acidic and basic forms of 80K, the phosphoproteins from untreated gels of mitogen-treated cells were excised in sections as indicated in Figure 2b, and subjected to tryptic peptide map analysis (Figure 4). Overall it was found that forms of 80K with similar pI from cells treated in different ways had similar phosphopeptide patterns. For example, the most basic form of 80K from bombesin-treated cells

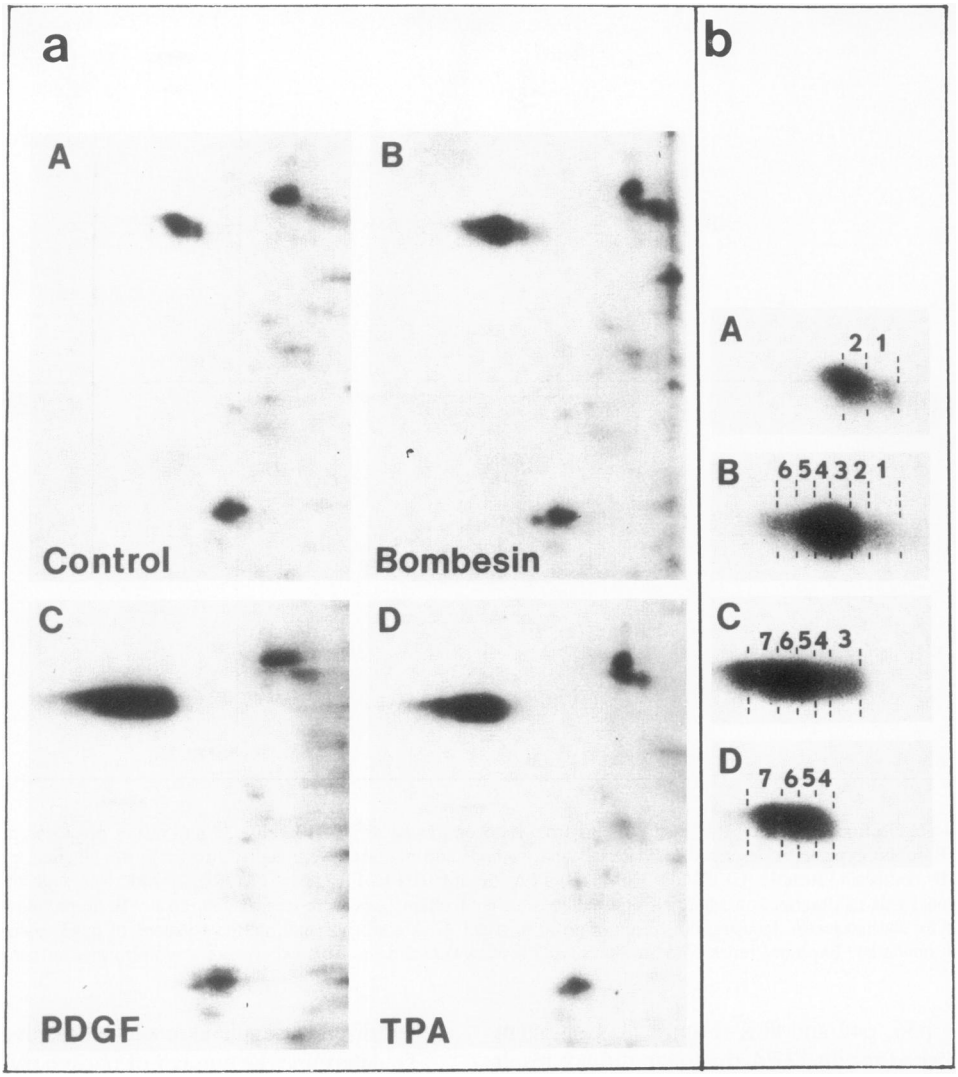

Fig. 2. The 80K region in two-dimensional gels from Swiss 3T3 cells. (**a**) Two-dimensional gels of [^{32}P]orthophosphate-labeled Swiss 3T3 cells were as described in Figure 1 except that the gels were dried before fixing. The higher mol. wt acidic regions of these gels including 80K and surrounding proteins are shown. (**A**) No addition, (**B**) bombesin, 10 nM; (**C**) PDGF, 1 nM; (**D**) TPA, 50 nM. Exposure time was 2 h with an intensifying screen. (**b**) The same autoradiograms as shown in (a) were enlarged and the region containing 80K was aligned vertically using the surrounding proteins. In addition, the sections that were excised and subjected to tryptic peptide mapping in Figure 4 are indicated by numbers.

(B 1) had a phosphopeptide pattern similar to the most basic form of 80K from untreated cells (Control 1). While many of the phosphopeptides were present in all the isoelectric forms of 80K, some differences were noted. The most basic form of 80K (Control 1 and B 1) lacked phosphopeptides 1, 3 and 5, whereas more acidic forms of the protein had a greater abundance of these three peptides. On moving toward the most acidic forms of 80K a loss of peptides 9 and 10 (B 6) and finally of peptides 6, 8 and 11 (TPA 7) was found. The data shown in Figure 4 confirm the observation made in Figure 3, namely, that when the whole group of 80K proteins are examined an increase in the amount of acidic forms results in an increase in the amounts of tryptic phosphopeptides 1, 3 and 5. Bombesin treatment resulted in a less complete conversion of basic to acidic forms of 80K than either PDGF or tumor promoter treatments (Figure 2a and b) and has accordingly less of peptides 1, 3 and 5 (Figure 3).

It remained possible that 80K represented protein kinase C itself, which is reported to have a mol. wt of 80 kd and be phosphorylated in intact cells (Fry *et al.*, 1985). This possibility was excluded by demonstrating that protein kinase C immunoprecipitated from [^{35}S]methionine-labeled murine cells did not co-migrate with 80K from TPA-treated Swiss 3T3 cells in two-dimensional gels (J.R.Woodgett and J.Meisenhelder, unpublished results).

Additional evidence that bombesin activates protein kinase C

The activation of protein kinase C has been measured by a number of indirect assays including stimulation of 80K phosphorylation (see above) and a decrease of [^{125}I]EGF binding to its receptor. Here we examined the [^{125}I]EGF binding capacity in parallel with the mitogenic and phosphorylation asssays (Table I and Figure 1). Dishes of cells were incubated for 60 min with mitogens and [^{125}I]EGF at 37°C and the amount of radioactivity associated with the cells after this period was measured (Table I). In agreement with previous reports all the mitogens tested caused a decrease in EGF binding (Brown *et al.*, 1979; Sinnett-Smith and Rozengurt, 1985; Brown *et al.*, 1984). Bombesin has been reported to be as effective as PDGF in stimulating phosphoinositide breakdown (Brown *et al.*, 1984). However, in our experiments bombesin does not appear as potent an activator of

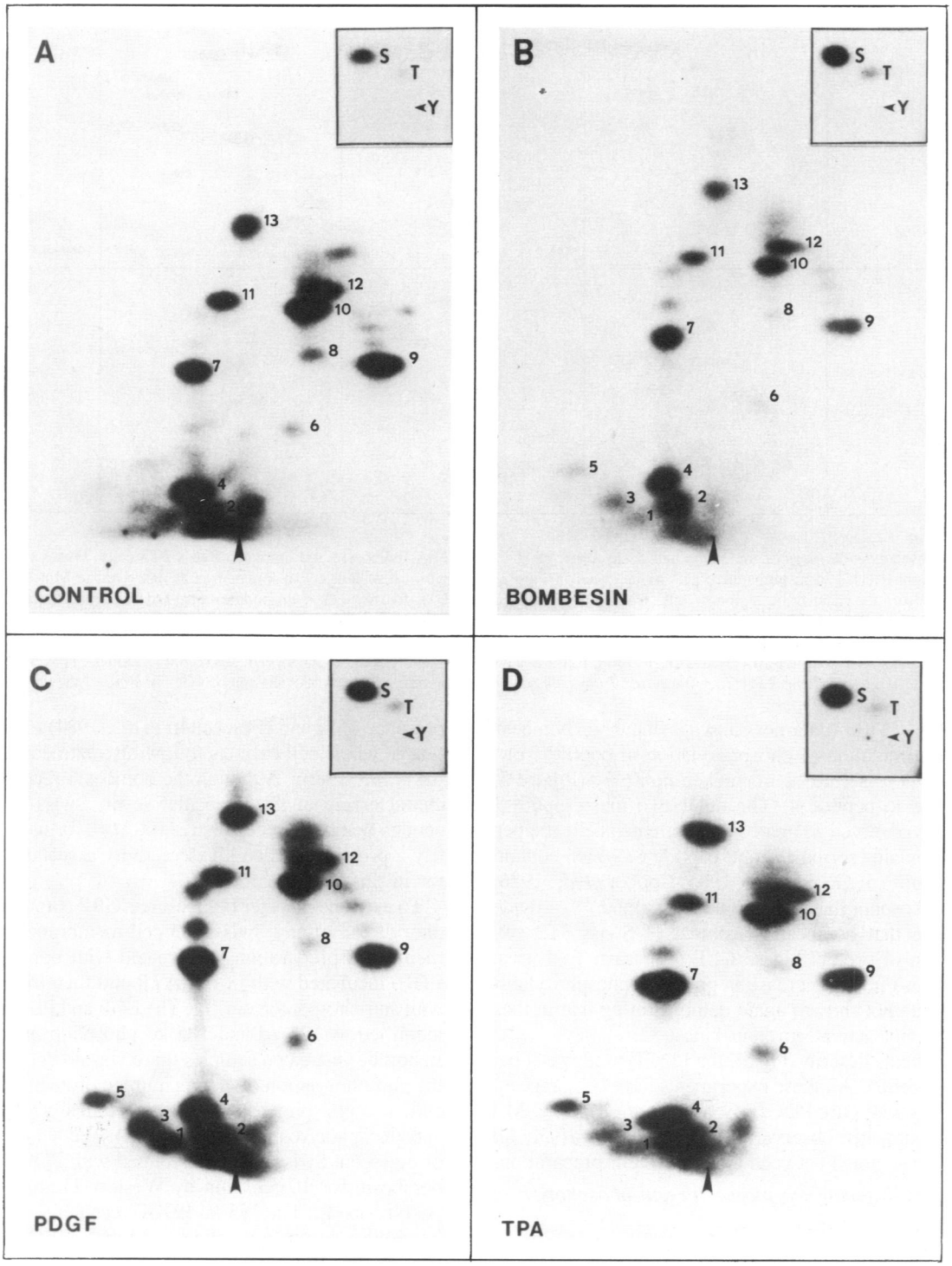

Fig. 3. Tryptic phosphopeptide maps and phosphoamino acid analysis of 80K from mitogen-treated Swiss 3T3 cells. The entire 80K area was excised from the two-dimensional preparative gels shown in Figure 2a and subjected to trypsin digestion and separation in two dimensions as described in Materials and methods. In each case the origin is marked (arrowhead). The peptide numbers correspond to those used in the schematic diagram in Figure 4. Electrophoresis on 100 μm cellulose thin-layer plates was performed in the first dimension at pH 8.9 for 20 min at 1 kV with the anode on the left. For each sample, 80K from 2–4 gels was pooled. Cerenkov c.p.m. analysed in each case were: (**A**) untreated cells, 1080 c.p.m.; (**B**) bombesin (10 nM), 420 c.p.m.; (**C**) PDGF (1 nM), 1330 c.p.m.; (**D**) TPA (50 nM), 1050 c.p.m. **Inset**: 1/8th of each 80K sample described above was subjected to phosphoamino acid analysis prior to trypsin digestion. The position of phosphoserine (**S**), phosphothreonine (**T**) and phosphotyrosine (**Y**) are indicated. Exposure time with an intensifying screen was 5 days.

protein kinase C as either PDGF or TPA, based on the degree of inhibition of EGF binding, as well as the lower level of p42 phosphorylation (Figure 1) and the fewer acidic forms of 80K generated (Figure 4; see Discussion).

Finally, as neither the 80K phosphorylation nor EGF binding assays provide direct evidence for protein kinase C activation, we examined the phosphorylation of pp60$^{c\text{-}src}$ in Swiss 3T3 cells in response to mitogen treatments. The incubation of purified protein kinase C with pp60$^{c\text{-}src}$ and [γ-^{32}P]ATP *in vitro* or the treatment of cells with tumor promoters *in vivo* both result in a novel phosphorylation event on pp60$^{c\text{-}src}$ (Gould *et al.*, 1985; Gentry *et al.*, 1986). Treatment of Swiss 3T3 cells for 10 min

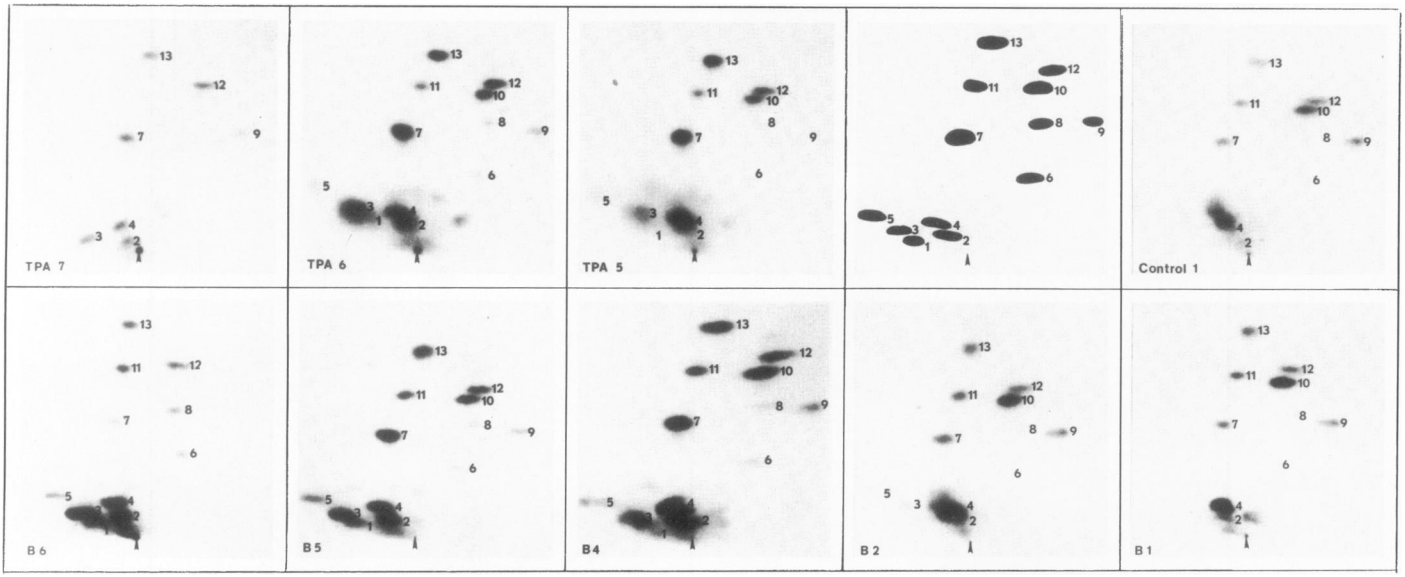

Fig. 4. Tryptic phosphopeptide maps of the acidic and basic forms of 80K from TPA, bombesin and untreated Swiss 3T3 cells. Forms of 80K with different isoelectric points were excised form preparative gels as indicated in Figure 2b. Trypsin digestion and mapping was as described in Materials and methods and in Figure 3. Maps from the most basic region of 80K from control cells (**Control 1**), five regions from bombesin-treated cells ranging from basic to acidic (**B 1, B 2, B 3, B 4** and **B 6**) and three regions from TPA-treated cells (**TPA 5, TPA 6** and **TPA 7**) are shown. For each sample 80K regions from six preparative two-dimensional gels were pooled. Cerenkov c.p.m. analysed and exposure time with an intensifying screen were: control 1, 400 c.p.m., 7 days; B 1, 210 c.p.m., 7 days; B 2, 620 c.p.m., 3 days; B 3, 780 c.p.m., 3 days; B 4, 1190 c.p.m., 3 days; B 6, 430 c.p.m., 7 days; TPA 5, 1000 c.p.m., 3 days; TPA 6, 1080 c.p.m., 3 days; TPA 7, 350 c.p.m., 7 days. A schematic diagram of the tryptic phosphopeptides of 80K is shown.

with either TPA, PDBu (data not shown), PDGF or bombesin caused a large stimulation of phosphorylation of pp60$^{c\text{-}src}$ on a residue equivalent to serine 12 in chicken pp60$^{c\text{-}src}$ (Figure 5), which gives rise to peptide 4. The other two major pp60$^{c\text{-}src}$ phosphopeptides observed in treated and untreated cells are peptide 1, which contains serine 17, and peptide 6, which contains tyrosine 527 (Figure 5; Gould *et al.*, 1985; Cooper *et al.*, 1986). The increased phosphorylation of serine 12 in pp60$^{c\text{-}src}$ provides further evidence that bombesin treatment of Swiss 3T3 cells causes activation of protein kinase C. By contrast, EGF treatment did not result in any increase in pp60$^{c\text{-}src}$ phosphorylation on serine 12 (data not shown) again demonstrating that in these cells EGF does not activate protein kinase C.

In the experiments described here, the PDGF used was a partially pure preparation. All these experiments have been repeated at different times with pure PDGF (Cooper *et al.*, 1982; C.M.I. and K.L.G. unpublished observations). In no case was any difference in response noted between the two PDGF preparations.

Detection of growth factor receptors with anti-phosphotyrosine antibodies

The failure to detect an increase in the level of phosphotyrosine in total cellular proteins or in known protein-tyrosine kinase substrates does not necessarily preclude the possibility that the bombesin receptor has intrinsic protein-tyrosine kinase activity, since the bombesin receptor might have specificities different to that of other known receptor protein-tyrosine kinases. Growth factor receptors, such as the PDGF receptor, which have associated protein-tyrosine kinase activity are phosphorylated on tyrosine in the presence of their ligand. None of the techniques employed so far were designed specifically to detect autophosphorylation of the putative bombesin receptor. In the absence of specific anti-receptor antibodies, autophosphorylation of growth factor receptors can be detected using anti-phosphotyrosine antibodies either by immunoprecipitation of membrane preparations phosphorylated *in vitro* by [γ-^{32}P]ATP in the presence of ligand (Frackelton *et al.*, 1984) or by Western blotting of whole cell extracts following treatment with ligand (Zippel *et al.*, 1986). Although the bombesin receptor has not been characterized at the molecular level, Swiss 3T3 cells contain enough bombesin receptors ($\sim$100 000) for us to attempt to identify a protein-tyrosine kinase activity associated with its receptor in this way.

To examine whether bombesin or GRP stimulate tyrosine phosphorylation of any Swiss 3T3 cell membrane proteins, a crude membrane preparation was treated with bombesin, PDGF, or EGF, incubated with [γ-^{32}P]ATP and then immunoprecipitated with anti-phosphotyrosine Ig. The EGF and PDGF receptors were identified as 175 kd and 185 kd phosphoproteins respectively. In bombesin-treated samples there was no detectable increase in the phosphorylation of any membrane proteins (data not shown), and no phosphoprotein was specifically precipitated with anti-phosphotyrosine Ig (Figure 6a). We also analysed extracts of quiescent Swiss 3T3 cells treated with PDGF, EGF, GRP or bombesin for 10–30 min by Western blotting with anti-phosphotyrosine Ig. The 185 kd PDGF receptor was readily detected, in addition to proteins of 130, 70 and 33 kd (marked with arrowheads in Figure 6), which were specific to PDGF-treated cells. The 175 kd EGF receptor was detectable in EGF-treated cells, but less apparent than the PDGF receptor. A protein of 48 kd was preferentially found in EGF-treated cells (marked by arrowhead in Figure 6). In all the mitogen-treated samples proteins of 38 and 90 kd were observed, which were absent or less intense in control samples. However, there were no striking bands which were unique to extracts of bombesin- or GRP-treated cells (Figure 6b).

Discussion

In examining phosphorylation events induced by bombesin and GRP treatment of Swiss 3T3 cells, the most obvious changes are those which can be attributed to the activation of protein kinase

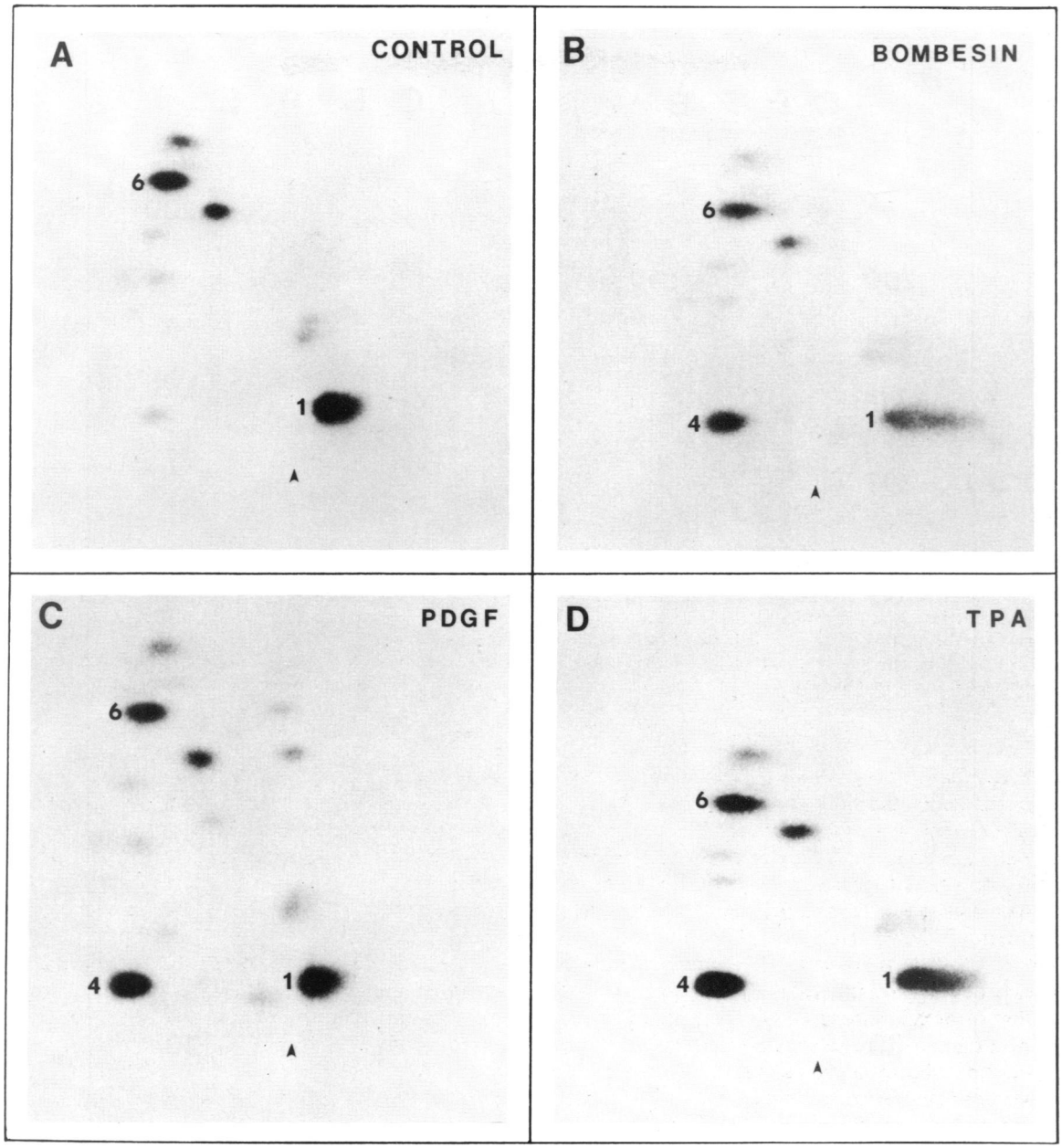

Fig. 5. Tryptic phosphopeptide maps of pp60$^{c\text{-}src}$ from mitogen-treated Swiss 3T3 cells. pp60$^{c\text{-}src}$ was isolated from treated or untreated Swiss 3T3 cells by immunoprecipitation with MAb 327 (Lipsich *et al.*, 1983), subjected to tryptic digestion and separated in two dimensions as described by Gould *et al.* (1985). In each case the origin is marked (arrowhead). Cells had been labeled with 2.5 mCi/ml [^{32}P]orthophosphate for 18 h and treated for 10 min with 140 nM 4-β-phorbol, (**B**) 10 nM bombesin, (**C**) 1 nM PDGF or (**D**) 75 nM TPA. Approximately 200 Cerenkov c.p.m. were analysed in each case. Exposure time with an intensifying screen was 5 days.

C. Tumor promoters, which are thought to exert their mitogenic effects by binding to and activating protein kinase C (reviewed in Nishizuka, 1984; Ashendel, 1985), induce a number of cellular events, including the indirect inhibition of EGF binding to its cell surface receptor (Brown *et al.*, 1979; Sinnett-Smith and Rozengurt, 1985) and the increased phosphorylation of 80K (Rozengurt *et al.*, 1983; Blackshear *et al.*, 1985, 1986; Rodriguez-Pena and Rozengurt, 1986). Here we examined the effect of bombesin and GRP on these cellular events and found that by both criteria the bombesin peptides activated protein kinase C (Table I; Figures 1 and 2), thus confirming the results of Zachary *et al.* (1986). In addition, we employed a more direct assay for the activation of protein kinase C, namely the stimulation of serine 12 phosphorylation of pp60$^{c\text{-}src}$ (Gould *et al.*, 1985) and showed that bombesin was able to induce this phosphorylation event (Figure 5). In the course of these studies we confirmed that PDGF treatment, in addition to stimulating tyrosine phosphorylation, activates protein kinase C in Swiss 3T3 cells. By contrast we found no evidence that EGF induces stimulation of protein kinase C. EGF treatment failed to increase serine 12 phosphorylation of pp60$^{c\text{-}src}$ (data not shown) and does not stimulate 80K phosphorylation (Rozengurt *et al.*, 1983; Bishop *et al.*, 1985; Blackshear *et al.*, 1985; Figure 1). We routinely observed that neither bombesin nor GRP was as an efficient activator of protein kinase C as the tumor promoters, although GRP was reproducibly slightly more effective than bombesin. Tumor promoters may be more potent because they are not as readily metabolized as the DAG endogenously produced in response to bombesin and GRP.

Our investigation of the group of 80 kd phosphoproteins extends the work of others. Blackshear *et al.* (1985, 1986) demonstrated that 80K from tumor promoter, PDGF or synthetic

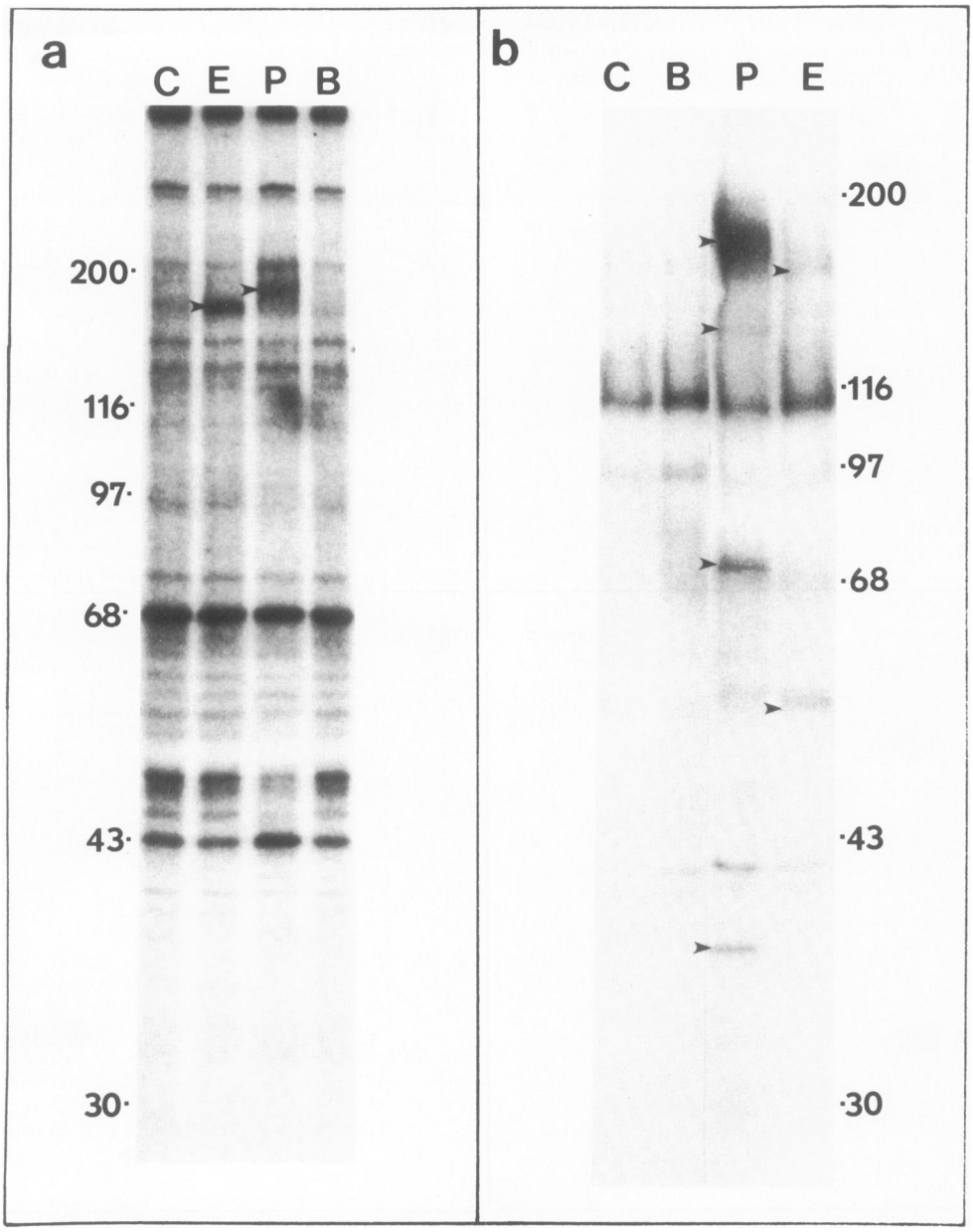

Fig. 6. Detection of growth factor receptors with anti-phosphotyrosine antibodies. **(a)** For each sample a crude membrane preparation from about 1×10^7 Swiss 3T3 cells was resuspended in 20 μl 10 mM Hepes (pH 7.4) and incubated for 15 min at 4°C with mitogens to give a concentration of 100 nM EGF (**E**), 100 nM PDGF (**P**), 1 μM bombesin (**B**) or an equivalent volume (10 μl) of 1 mM acetic acid containing 1 mg/ml BSA as control (**C**) and then for 10 min at 30°C with 50 μCi [γ-^{32}P]ATP as described in Materials and methods. The membranes were solubilized in RIPA buffer and immunoprecipitated with anti-phosphotyrosine antibodies as described in Materials and methods. One-half of each sample was resolved on a 10% SDS-polyacrylamide gel. Exposure time with intensifying screen at −70°C was 2 days. **(b)** Quiescent Swiss 3T3 cells in 35 mm dishes were treated with 10 nM bombesin (**B**), 1 nM PDGF (**P**), 5 nM EGF (**E**) for 15 min or left untreated (**C**) and then lysed in SDS sample buffer. One-tenth of each sample was resolved on a 10% SDS-polyacrylamide gel and immunoblotted as described in Materials and methods. Exposure time was 2 days. Mol. wt markers were myosin (200 kd), β-galactosidase (116 kd), phosphorylase (97 kd), bovine serum albumin (68 kd), ovalbumin (43 kd) and carbonic anhydrase (30 kd). Arrowheads indicate proteins referred to in the text.

DAG-treated 3T3 cells was phosphorylated predominantly on serine residues, and resolved into several charge species. Rozengurt and colleagues showed that bombesin, like tumor promoters, increased 80K phosphorylation (Zachary *et al.*, 1986) and that protein kinase C is very likely to mediate this directly (Rodriguez-Pena and Rozengurt, 1986). By phosphoamino acid analysis and tryptic peptide mapping we found that 80K was basally phosphorylated on serine and threonine residues. Mitogen treatment caused additional phosphorylation on serine residues, which resulted in the conversion to more acidic forms. Seven isoelectric forms of 80K could be resolved as discrete vertical bands. Up to 13 phosphopeptides were present in pooled 80K preparations. Because of the possibility of incomplete tryptic digestion, it is unclear exactly how many phosphorylation sites there are. If the charge isomers represent the addition of single phosphate residues, then there can be as many as seven phosphates per 80K molecule, implying there are at least seven distinct phosphorylation sites. Some differences in tryptic phosphopeptides content between the isoforms was evident in going from basic to acidic, but there was no evidence that these phosphorylations must occur in a strictly ordered sequence. Thus the most basic form of 80K, which probably contains a single

phosphate, can be phosphorylated at one of many sites. These results are consistent with there being a single 80K protein.

The PDGF and EGF receptors have intrinsic ligand-stimulated protein-tyrosine kinase activities. The fact that the response of Swiss 3T3 cells to bombesin has similarities to both the EGF and PDGF responses, raises the question of whether the bombesin receptor might likewise have protein-tyrosine kinase activity. We failed to detect a marked increase in tyrosine phosphorylation of cellular proteins following bombesin treatment of quiescent cells, but the methods we used to detect such a stimulation have limitations. For instance, only a minor increase in total phosphotyrosine levels is detected in cells following EGF treatment unless the cells have unusually high levels of EGF receptors such as A431 cells (Hunter and Cooper, 1981). Thus even though Swiss 3T3 cells possess about 85 000–100 000 EGF receptors (Brown *et al.*, 1984; Zachary *et al.*, 1986) and are mitogenically responsive to EGF (Table I), we did not observe a significant increase in phosphotyrosine levels following EGF treatment. Two-dimensional gel analysis of phosphoproteins for phosphotyrosine-containing proteins also has shortcomings, which we have discussed elsewhere (Cooper and Hunter, 1983). For example, despite the relatively large increase in total phosphotyrosine in PDGF-treated cells, the only phosphotyrosine-containing proteins detected by this technique are p42 and p36 (Cooper *et al.*, 1982).

We were also unable to obtain direct evidence that the bombesin/GRP receptor has a ligand-stimulated protein-tyrosine kinase activity, by using anti-phosphotyrosine antibodies either for immunoprecipitation of *in vitro* phosphorylated membrane preparations or for Western blotting of whole-cell extracts, techniques which allowed detection of the PDGF receptor. This contrasts with a recent study by Comoglio and co-workers (Cirillo *et al.*, 1986), where a 105 kd protein was observed by Western blotting extracts of bombesin-treated Swiss 3T3 cells with anti-phosphotyrosine Ig. These workers were also able to immunoprecipitate a protein of this size, whose phosphorylation on tyrosine *in vitro* was stimulated by bombesin. Their results suggest that the bombesin receptor is a protein of 105 kd with ligand-stimulated protein-tyrosine kinase activity. Our failure to detect such a protein may be attributable to the anti-phosphotyrosine antibody preparation we used, since there is documented variability in the affinity of anti-phosphotyrosine antibodies towards individual phosphotyrosine-containing proteins.

If the bombesin receptor has protein-tyrosine kinase activity, our analysis of tyrosine phosphorylation events in the intact cell suggests that the bombesin receptor either has a lower specific activity or else has a much more restricted substrate specificity than the PDGF receptor protein-tyrosine kinase. In this respect the properties of the bombesin receptor are more akin to those of the EGF receptor, since treatment by neither factor results in an elevation in whole-cell phosphotyrosine levels. However, a major difference in the mechanism of action of the bombesin and EGF receptors is observed; while PDGF and bombesin are potent activators of PI turnover in Swiss 3T3 cells (Habenicht *et al.*, 1981; Brown *et al.*, 1984), EGF treatment of most cells, including Swiss 3T3 cells, does not result in the activation of protein kinase C (Bishop *et al.*, 1985; Blackshear *et al.*, 1985; Habenicht *et al.*, 1981; Coughlin *et al.*, 1985). Clearly one needs to identify the substrate for the PDGF receptor protein-tyrosine kinase which when phosphorylated is responsible for the increase in DAG level, and determine whether this is also phosphorylated in bombesin-treated cells, or whether bombesin activates PI turnover in another manner. The fact that PDGF, EGF and probably bombesin all stimulate tyrosine phosphorylation upon binding to their receptors and yet induce distinct phosphorylation responses implies that there are multiple signal effector pathways available for the receptor protein-tyrosine kinases.

To understand the role of bombesin and related peptides in growth control, an important question is whether the bombesin receptor on Swiss 3T3 cells is the same as that on small cell lung carcinoma cells. A comparison of the bombesin and bombesin-related peptide binding to these cell lines suggests that the same receptor is utilized in both cases (Zachary and Rozengurt, 1985; Moody *et al.*, 1985). [^{125}I]GRP or an analogue of bombesin containing an iodinatable tyrosine residue, [Tyr^4–^{125}I]bombesin, bind to a single class of high affinity sites (K_D = 0.5 nM). In both cell types this binding is displaceable with bombesin-related peptides which have mitogenic activity, e.g., bombesin, (Tyr^4) bombesin and GRP, but not by inactive peptides such as vasopressin, vasoactive intestinal peptide or substance P. Thus, an understanding of the mechanism by which the bombesin/GRP receptor on Swiss 3T3 cells transmits its mitogenic signal may well provide an insight into the role of the bombesin-related peptides in the autocrine stimulation of small cell lung carcinomas.

Materials and methods

Materials

PDGF (~0.4% pure) was a gift from Elaine Raines (University of Washington, Seattle, WA; Raines and Ross, 1982). EGF was obtained from S.Potter (The Salk Institute; Savage and Cohen, 1972). 4-β-Phorbol, 4-β-phorbol-12,13-dibutyrate (PDBu) and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma Chemicals. Bombesin and GRP were a gift from Jean Rivier (The Salk Institute; Rivier and Brown, 1978). Peptides were dissolved in water or Dulbecco-Vogt modified Eagle's medium (DMEM) and stored as 1 mM stock solutions at −20°C. Concentrations of mitogens used in each assay are given in the figure legends.

Cells

A bombesin-responsive Swiss 3T3 cell clone was obtained from Enrique Rozengurt (ICRF, London), and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Unless otherwise stated confluent, quiescent cells were prepared for assays by seeding 2.5 × 10^4 cells into a 35 mm dish in DMEM supplemented with 10% FBS and culturing for 6–7 days. For metabolic labeling, the medium was then aspirated and the cells cultured for a further 18 h in 1 ml of phosphate-free DMEM containing 5% (v/v) DMEM and 2 mCi of [^{32}P]orthophosphate (ICN). Mitogens were added for 15 min prior to lysis.

A crude membrane preparation was made from Swiss 3T3 cells as follows: cells (~80% confluent) were scraped from 150 mm dishes at 4°C in 2 ml of 5 mM Hepes (pH 7.4), 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% aprotinin, 0.1 mM PMSF, 4 μg/ml SBTI, 4 μg/ml leupeptin and homogenized with 25 strokes in a Dounce homogenizer using a tight fitting pestle. The homogenate was centrifuged at 200 *g* for 5 mins to remove remaining whole cells, nuclei and cell debris, and then at 20 000 *g* for 60 min. The supernatant was discarded and the membrane pellet stored in liquid N_2.

Assays

Whole cell phosphoamino acid analysis. This was performed according to Sefton *et al.* (1980).

Two-dimensional gel electrophoresis. This was performed using the method of Garrels (1979) modified by Cooper and Hunter (1981) except that isoelectric focusing was with pH 3.5–10 ampholytes (LKB). The gels were fixed, dried and rehydrated in 1 M KOH, incubated at 55°C for 2 h, neutralized, and dried again (Cooper and Hunter, 1981). Preparative gels for 80K were rinsed for 30 min in water with mixed-bed ion exchange resin (Amberlite) and dried without fixing.

Inhibition of EGF binding. This was assayed according to Brown *et al.* (1984). Essentially dishes of cells were cultured overnight in labeling medium without [^{32}P]orthophosphate and then incubated at 37°C for 60 min in 1 ml DMEM containing mitogens and 27 000 c.p.m. [^{125}I]EGF (NEN; 175 μCi/μg). After washing in ice-cold DMEM, the cells were extracted with 0.5 M NaOH and counted in a γ-counter.

Measurement of [^{3}H]thymidine incorporation. Dishes of cells were cultured for a further 24 h with 2 ml of DMEM. Mitogens together with 1 μg/ml insulin were added for another 24 h, and the cells were labeled with 2 μCi/ml [methyl-^{3}H]thymidine (6.7 Ci/mmol, NEN) for the final 6 h. Cells were then fixed with methanol:acetic acid (3:1, v/v), washed extensively with 10% trichloroacetic acid

(TCA) at 4°C, rinsed with PBS and solubilized in 1 ml 0.2 M NaOH, 0.5 ml was counted in 10 ml of aqueous scintillation fluid.

Peptide mapping and phosphoamino acid analysis. 80K phosphoproteins were excised from the preparative gels and then protein was subjected to phosphoamino acid analysis or tryptic peptide mapping as described by Hunter and Sefton (1980). The ^{32}P-labeling of pp60$^{c\text{-}src}$ *in vivo* and its tryptic peptide mapping have been previously described (Gould *et al.*, 1985). In this experiment mitogen treatments were for 10 min prior to lysis.

Western blotting. Dishes of cells were cultured for a further 24 h in DMEM alone and then treated with mitogens for 10 to 30 min before rinsing once with cold PBS and lysing the cells in 0.3 ml boiling Laemmli sample buffer. The samples were boiled for 5 min, sheared by repeated passage through a 27 gauge needle, reboiled for 20 min and equal volumes were resolved on 10% SDS–polyacrylamide gels. Proteins were transferred to 0.45 μm nitrocellulose (Schleicher and Schuell) in a BioRad apparatus using a Tris–glycine buffer containing 20% methanol at a current of 40 V for 90 min. The blot was then blocked overnight in rinse buffer (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) containing 5% BSA and 1% ovalbumin and probed for 2 h in the same buffer containing 4 μg/ml affinity-purified anti-phosphotyrosine Ig generated by the immunization of rabbits with poly[phosphotyrosine.Ala.Gly] covalently linked to keyhold limpet hemocyanin (a kind gift of Mark Kamps, Salk Institute). The blot was then washed twice in rinse buffer, once in rinse buffer containing 0.05% NP-40, and then twice more in rinse buffer for 10 min each. The blot was incubated with [^{125}I]-protein A for 1 h to decorate bound antibody, washed as above, dried and exposed to film.

Immunoprecipitation of phosphorylated membrane proteins. Crude membrane preparations (see above) from ~1 × 10^7 cells were resuspended in 20 μl of 10 mM Hepes (pH 7.4), incubated for 15 min at 4°C with mitogens and then phosphorylated *in vitro* for 10 min at 30°C with 50 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) in 25 μl of 20 mM Pipes (pH 7.4), 10 mM $MnCl_2$, 4 μM unlabeled ATP (final concentration). Membranes were then solubilized in 0.5 ml RIPA buffer (Sefton *et al.*, 1978) containing 50 mM Tris, pH 7.4 instead of phosphate buffer and supplemented with 5 mM EDTA; 100 μM Na_3VO_4; 50 mM NaF. The lysate was then clarified by centrifugation at 20 000 *g* for 60 min and immunoprecipitated according to Sefton *et al.* (1978) using anti-phosphotyrosine Ig affinity-purified from a rabbit antiserum raised against phosphotyrosine coupled to BSA (obtained from Mark Kamps, The Salk Institute).

Autoradiography. This was performed using Kodak XAR film, which where indicated was pre-sensitized and exposed at −70°C with an intensifying screen.

Acknowledgements

We are extremely grateful to Enrique Rozengurt for providing us with the bombesin-responsive clone of Swiss 3T3 cells and also for communicating results prior to publication, and to Paolo Comoglio for informing us of experiments concerning the nature of the bombesin receptor prior to publication. We thank Jean Rivier for supplying bombesin and gastrin-releasing peptide and Mark Kamps for the affinity-purified anti-phosphotyrosine antibodies. This work was supported by Public Health Service Grant Numbers CA 17096, CA 28458 and CA 39780 from the National Cancer Institute. C.M.I. was a recipient of a fellowship from the Damon Runyon–Walter Winchell Cancer Fund (DRG-775). K.D.B. was a recipient of a grant from the Nuffield Foundation and is grateful to Bob Holley at the Salk Institute for providing support and laboratory space. K.L.G. and S.J.G. were supported by National Institutes of Health predoctoral training grants to the University of California at San Diego.

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Received on 2 April 1986; revised on 1 September 1986