Differential Protein Phosphorylation in Induction of Thyroid Cell Proliferation by Thyrotropin, Epidermal Growth Factor, or Phorbol Ester

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Protein phosphorylation was studied in primary cultures of thyroid epithelial cells after the addition of different mitogens: thyrotropin (TSH) acting through cyclic AMP, epidermal growth factor (EGF), or 12-O-tetradecanoylphorbol-13-acetate (TPA). EGF or TPA increased the phosphorylation of five common polypeptides. Among these, two 42-kilodalton proteins contained phosphotyrosine and phosphoserine with or without phosphothreonine. Their characteristics suggested that they are similar to the two 42-kilodalton target proteins for tyrosine protein phosphorylation demonstrated in fibroblasts in response to mitogens. No common phosphorylated proteins were detected in TSH-treated cells and in EGF- or TPA-treated cells. The differences in the protein phosphorylation patterns in response to TSH, EGF, and TPA suggested that the newly emerging cyclic AMP-mediated mitogenic pathway is distinct from the better known growth factor- and tumor promoter-induced pathways.

The biochemical steps involved in the pathway leading a eucaryotic cell from quiescence to DNA synthesis after the application of an exogenous mitogenic stimulus have been primarily studied in fibroblasts or cell lines. Two main pathways have been delineated (2, 6). In one pathway, certain growth factors, such as epidermal growth factor (EGF), insulin, and type I insulinlike growth factor stimulate a receptor at the exterior of the cell and its intracellular tyrosine kinase activity.

In the alternative pathway, growth factors such as α -thrombin or vasopressin stimulate the hydrolysis of inositol lipids. The extracellular signal activates a receptor which through a GTP-binding transducing protein activates a phospholipase C which releases in the cell diacylglycerol and inositol 1,4,5-triphosphate (IP_3) as intracellular signals. IP_3 provokes the release of Ca^{2+} from the endoplasmic reticulum. Depending on the cell type, Ca^{2+} and diacylglycerol together or separately induce DNA synthesis. The role of diacylglycerol as an initiator of proliferation has been demonstrated in many cell types by using phorbol esters as probes of its target enzyme, protein kinase C (28, 38). The receptor for platelet-derived growth factor seems to activate both signal pathways. This could also be the case for the bombesin receptor, although its tyrosine kinase activity is still debated (7, 19).

These two pathways, which are clearly distinct as shown by their differential sensitivity to a pretreatment with pertussis toxin (6), may join or at least overlap at some point(s) beyond the initial signal receptor interaction. Indeed, although they involve the stimulation of distinct protein kinases at the membrane level, they elicit the phosphorylation of a common set of 42-kilodalton (kDa) proteins on tyrosine, threonine, and/or serine residues. This phosphorylation is a general response to all mitogens acting through either pathway, at least in fibroblasts (9, 13, 16, 20, 21, 27). Moreover, these 42-kDa proteins are the only substrates phosphory-

lated on tyrosine detected so far in most types of fibroblasts stimulated to proliferate by these mitogens.

For several years, cyclic AMP has been shown to induce cell growth in several types of differentiated cells (4). Thyrotropin (TSH), acting through cyclic AMP, induces DNA synthesis in quiescent dog thyroid cells (34). The dog thyroid cell also responds to the two other types of mitogenic signals, EGF and 12-O-tetradecanoylphorbol-13-acetate (TPA) (33, 35). All three mitogenic pathways involve the stimulation of distinct protein kinases. The purpose of this work was to delineate in this type of cell the pattern of protein phosphorylation characterizing each pathway and, in particular, to assess the phosphorylation of the 42-kDa proteins in the cyclic AMP-dependent pathway.

MATERIALS AND METHODS

Cell culture. Dog thyroid cells were cultured in a serum-free defined medium as previously described (35). This culture medium contains Dulbecco modified Eagle medium-Hams F12-MCDB104 (2:1:1 [vol/vol/vol]; Flow Laboratories, Inc., McLean, Va.) with 2 mM glutamine, supplemented with 5 μ g of insulin per ml and 40 μ g of ascorbic acid per ml. Antibiotics (100 units of penicillin per ml, 100 μ g of streptomycin per ml) were also added.

Radioactive labeling. After 4 days of culture, the cells were incubated for 8 or 20 h in the labeling medium Eagle minimal essential medium containing 9 μ M KH_2PO_4 , 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5 μ g of insulin per ml, 2 mM glutamine, and 1 or 3 mCi of $^{32}PO_4$ (carrier-free; Amersham Corp., Arlington Heights, Ill.) per ml. The concentrations of TPA (Sigma Chemical Co., St. Louis, Mo.), EGF (Collaborative Research, Inc., Waltham, Mass.), TSH (Armour Pharmaceutical Co.), and forskolin (Calbiochem-Behring, La Jolla, Calif.) used were those required for maximal stimulation of proliferation of dog thyroid cells (32, 33).

DNA synthesis. DNA synthesis was estimated directly in the petri dishes by determining the frequency of the [3H] thymidine-labeled nuclei by autoradiography. The cells were

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cultured for 4 days and then incubated for 8 or 20 h in the labeling medium lacking $^{32}\text{PO}_4$. The cells were further cultured for 48 h in the culture medium supplemented with [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$, 30 μM ; Amersham), deoxycytidine (100 μM), and the mitogenic agent(s) tested (34). The cells were fixed with methanol. After being extensively washed, they were stained with toluidine blue (1%) and the proportion of labeled nuclei was evaluated by counting at least 1,000 nuclei from different microscopic fields.

Sample preparation. After the incubation, the cells were briefly rinsed at room temperature with the culture medium and 250 μl of lysis buffer (29) was added to each petri dish. The lysed cells were centrifuged in a Beckman L75B centrifuge at 20°C in a 50 Ti rotor at 100,000 $\times g$ for 1 h, and the pellet was discarded.

Gel electrophoresis. Two-dimensional gel electrophoresis was performed as described previously (24). Proteins were separated by isoelectric focusing on cylindrical gels in the first dimension with 1.6% Servalytes pH 5 to 7 plus 0.4% Servalytes pH 2 to 11 or 1.6% Servalytes pH 4 to 6 plus 0.4% Servalytes pH 3 to 10 (Serva, Heidelberg, Federal Republic of Germany) and according to molecular mass on sodium dodecyl sulfate-linear gradient (6 to 16%) polyacrylamide slab gels in the second dimension. The dried gels were exposed to Kodak XAR5 films for a period calculated to reach a total of 1.2×10^{10} disintegrations (about 1 day). Autoradiographs are shown with acidic proteins on the left. Silver staining of the gels was performed as described previously (25). The sensitivity of the method is 0.02 ng/mm 2 .

Quantitative estimation of stimulation of phosphorylation. The area of the two-dimensional gel corresponding to the spot of interest seen on the autoradiograph was excised and swollen in 200 μl of H $_2\text{O}$ overnight. The protein was then solubilized in 1 ml of Soluene 350 (Packard Instrument Co., Inc., Rockville, Md.) for 2 h at 40°C, and its radioactivity was measured in a liquid scintillation counter after the addition of 12 ml of Insta Fluor (Packard).

Electroblotting technique. Electrophoretic transfer of proteins after electrophoresis was performed following the procedure of New England Nuclear Corp. (Boston, Mass.) based on techniques described by Towbin et al. (41) and Burnette (5). The transfer was done in a Bio-Rad Transblot cell for 20 h at 4°C and 7.5 V/cm. The transfer membrane was either nitrocellulose (PH 79; Schleicher & Schuell, Inc., Keene, N.H.) or nylon (Gene Screen; New England Nuclear Corp.). In both cases, the transfer buffer was 20 mM Tris–154 mM glycine–20% methanol.

Alkali treatment. After electrophoretic transfer of the proteins to nylon membranes, the sheets were treated with alkali in 1 N NaOH at 55°C for 2 h as previously described (8). The dried membranes were exposed to Kodak XAR5 film for a period calculated to reach a total of 10^{11} disintegrations (about 13 days).

Phosphoamino acid analysis. The proteins were transferred after electrophoresis to nitrocellulose membranes, excised, and eluted from the membranes by overnight incubation at 37°C in 50% pyridine–100 mM ammonium acetate (pH 8.9) as described previously (30). When phosphoamino acid analysis was performed on total proteins, these proteins were isolated as described previously (39). The proteins were hydrolyzed in 6 N HCl at 110°C for 1 h. Carrier phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) were then added to the sample, and the phosphoamino acids were separated by two-dimensional electrophoresis on cellulose plates as described previously (12). One or four

TABLE 1. Effect of mitogen treatment on [^3H]thymidine incorporation into the nuclei a

Expt b	Treatment	% [^3H]thymidine-labeled nuclei c
1	Control	2.1 $\pm$ 0.1
	TPA (50 ng/ml)	9.8 $\pm$ 1.2
2	Control	0.2 $\pm$ 0.2
	TPA (50 ng/ml)	10.5 $\pm$ 0.7
	EGF (50 ng/ml)	16.7 $\pm$ 2.5
3	Control	0.7 $\pm$ 0.0
	EGF (50 ng/ml)	21.0 $\pm$ 3.4
4	Control	3.6 $\pm$ 0.5
	TSH (1 mU/ml)	26.5 $\pm$ 0.7

a The cells were cultured for 4 days and incubated for 8 or 20 h in a phosphate-poor medium. The fluids were withdrawn, and [^3H]thymidine and the different mitogenic agents were added with the culture medium for 48 h as described in Materials and Methods.

b The experiments were performed on sister cultures corresponding to those shown in the following figures: experiment 1, Fig. 1 and 3; experiment 2, Fig. 2; experiment 3, Fig. 4; experiment 4, Fig. 5.

c Values are expressed as mean $\pm$ range of the percentage of labeled nuclei from duplicate petri dishes.

samples were applied on each plate. The carriers were revealed by ninhydrin staining (0.2% ninhydrin in acetone). The $^{32}\text{PO}_4$ -marked phosphoamino acids were detected by autoradiography obtained by exposure of the cellulose plate to a Kodak XAR5 film in the presence of a Siemens intensifying screen at -80°C for the required length of time. The amount of applied radioactivity on each plate was determined by Cerenkov counting of the extracted phosphoprotein before HCl hydrolysis.

RESULTS

Effects of TPA, EGF, and TSH on proliferation. Dog thyroid cells were cultured for 4 days without mitogenic agents to allow cell spreading and to reach a steady state. At this stage, in the absence of stimulating agents, dog thyroid cells remained quiescent; during the 48 h after the addition of [^3H]thymidine to the cell medium, virtually no incorporation into DNA was observed (about 2% of the cells were labeled). Addition of mitogenic agents induces, after a lag period of about 18 h, thymidine incorporation into the nuclei (35). The effects of TPA, EGF, and TSH on thymidine incorporation into DNA observed in this work are summarized in Table 1. The percentage of labeled nuclei was determined for separate petri dishes of each culture in which protein phosphorylation was studied.

Effect of TPA on protein phosphorylation. Figure 1A presents a two-dimensional gel electrophoresis pattern of total phosphopeptides extracted from quiescent dog thyroid cells after 8 h of incubation with $^{32}\text{PO}_4$. With the amount of radioactivity applied and the autoradiographic exposure time used, about 300 phosphoproteins were resolved. The weakest spots are not seen on the photographs. Figure 1B shows the pattern obtained when 50 ng of TPA per ml was added to the thyrocytes 15 min before the end of the incubation. The comparison of the two autoradiographs shows that although the majority of the phosphopeptides resolved are common for both incubation conditions, TPA modifies the phosphorylation of at least 25 proteins (in five of five experiments). The radioactivity is increased in 19 of these polypeptides (1 to 19, Table 2) and decreased in 6 (1' to 6', Table 3). The synthetic diglyceride *sn*-1,2-dioctanoyl glycerol (diC8) (100 $\mu\text{g}/\text{ml}$), a diacylglycerol analog which activates protein kinase C in intact cells (1, 14), faithfully reproduced the effect

FIG. 1. Two-dimensional gel analysis of phosphoproteins from thyroid cells treated with TPA. Quiescent dog thyroid cells were labeled with $^{32}\text{PO}_4$ for 8 h, and TPA was added 15 min before the end of the labeling period. Samples were subjected to electrophoresis in two dimensions as described in Materials and Methods. (A) No addition; (B) TPA, 50 ng/ml. The approximate molecular weight range for the second dimension is shown. The standard proteins used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme. The proteins whose phosphorylation was increased by TPA are identified with an arbitrary number (1 to 19) in panel B. The proteins whose phosphorylation was decreased in the presence of TPA are identified with an arbitrary number (1' to 6') in panel A.

of TPA on thyrocyte protein phosphorylation (Table 3). Increases in the phosphorylation state of the 19 proteins (1 to 19) were confirmed by excision of the proteins from the gel and by determination of their radioactivity (Table 2). A modification was considered to be significant when the counts incorporated in the TPA-stimulated phosphopeptide were at least twofold greater than the counts incorporated in the control protein. Samples were also analyzed by two-dimensional gel electrophoresis with Servalytes pH 4 to 6 rather than pH 5 to 7 for the isoelectric-focusing separation, but no additional changes in protein phosphorylation were seen (data not shown). Using one-dimensional gel electrophoresis of dog thyroid cells, it has been shown that TPA stimulates the phosphorylation of five proteins (28, 33, 42, 58, and 70 kDa) (17, 40), but their relationship to the TPA-phosphorylated proteins identified in this work is difficult to assess.

To detect tyrosine phosphorylation in TPA-stimulated phosphoproteins, total proteins were transferred after electrophoresis to nylon membranes and treated with 1 N NaOH for 1 h at 55°C. This alkali treatment hydrolyzes the majority of serine-bound phosphate groups. Comparison of Fig. 2A and 2B shows that TPA enhanced the alkali-stable phosphorylation of 10 proteins. To determine the nature of the phosphorylated residues in these proteins, we transferred total proteins after electrophoresis to nitrocellulose membranes instead of nylon membranes. The 10 phosphoproteins

of interest were then extracted and subjected to phosphoamino acid analysis. Six of these proteins contained only phosphoserine or phosphothreonine or both (data not shown). The phosphoamino acid analysis of the four others revealed the presence of phosphotyrosine in addition to phosphoserine with or without phosphothreonine. Figure 3 shows the phosphoamino acid composition of the pair of 42-kDa proteins (proteins 14 and 15). In quiescent cells, the phosphorylated amino acids present in these proteins were barely detectable. In TPA-treated cells, phosphorylation of tyrosine residues was stimulated in both 42-kDa proteins (Fig. 3B and 3D), while the phosphorylation of serine and threonine residues was stimulated only in the more acidic protein (Fig. 3B). The isoelectric pH of these two 42-kDa proteins was identical to that of the two 42-kDa proteins already described elsewhere (9, 20). Silver staining of the proteins after electrophoresis did not demonstrate the two 42-kDa proteins, suggesting low abundance (data not shown). The phosphoamino acid analysis of the two 45-kDa proteins (proteins 9 and 10) of TPA-treated cells showed that these two proteins contained phosphoserine and phosphothreonine and only trace amounts of phosphotyrosine (data not shown). These two proteins could be similar to the two 43-kDa proteins phosphorylated in response to TPA in human skin fibroblasts (20) and to the two 45-kDa proteins phosphorylated in response to EGF and platelet-derived growth factor in different Swiss 3T3 cell lines (9).

TABLE 2. Characteristics of the proteins whose phosphorylation is stimulated in response to TPA or diC8

Reference no. ^a	10 ³ M _r	³² P labeling of proteins (cpm) ^b		
		-TPA	+TPA	Fold increase
1	68	77 ± 11	220 ± 23	2.9
2	65	112 ± 7	402 ± 27	3.6
3	65	126 ± 0	268 ± 96	2.1
4	63	208 ± 5	474 ± 5	2.3
5	56	213 ± 11	578 ± 21	2.7
6	55	167 ± 21	506 ± 58	3.0
7	55	429 ± 13	1,234 ± 85	2.9
8	54	124 ± 16	249 ± 7	2.0
9	45	ND ^c	208 ± 13	
10	45	ND	224 ± 25	
11	43	78 ± 20	460 ± 23	6.0
12	43	44 ± 5	339 ± 26	7.7
13	43	84 ± 26	645 ± 51	7.7
14	42	129 ± 11	432 ± 1	3.3
15	42	92 ± 19	275 ± 10	3.0
16	34	21 ± 5	117 ± 1	3.0
17	34	39 ± 17	118 ± 20	5.9
18	28	993 ± 41	7,103 ± 168	7.2
19	15	381 ± 2	811 ± 18	2.1

^a Reference numbers identify the proteins shown in Fig. 1.^b Cells were labeled with ³²PO₄ for 8 h, and 50 ng of TPA per ml was added 15 min before the end of the culture period. Samples of the cell lysates containing equal amounts of counts per minute measured in phosphoproteins were subjected to electrophoresis. Radiolabeled proteins were visualized by autoradiography and excised from the gels, and the amounts of radioactivity were measured as described in Materials and Methods. An equivalent-sized gel piece that did not contain radiolabeled proteins and was therefore representative of the background level of radioactivity was excised from the gel and quantitated. This value (22 ± 3 cpm) was subtracted from the counts per minute measured in phosphoproteins. Values are expressed as mean ± range of duplicate assays.^c ND, Not detected.

Effect of EGF on protein phosphorylation. We first tested the effect of EGF on the tyrosine content of total cellular proteins. In agreement with results obtained in fibroblasts (9), EGF had little effect on the level of total cellular phosphotyrosine. The radioactivity found in phosphotyrosine expressed as a percentage of the total radioactivity recovered in phosphoamino acids was 0.306 ± 0.016 and $0.252 \pm 0.024\%$ for dog thyroid cells incubated for 15 min with or without EGF (50 ng/ml), respectively. Comparison of the phosphorylation pattern obtained when the thyroid cells were incubated in the presence or absence of EGF showed that EGF stimulates the phosphorylation of at least five polypeptides (in nine of nine experiments): a 28-kDa, two 42-kDa, and two 45-kDa proteins (Table 3). These five phosphoproteins were identical to five proteins whose phosphorylation was stimulated by TPA: proteins 18, 15, 14, 10, and 9 in Fig. 1B. No dephosphorylation of proteins phosphorylated in the basal state was detected after EGF treatment of thyroid cells. No additional changes in protein phosphorylation were observed with Servalytes pH 4 to 6 rather than pH 5 to 7 for the isoelectric-focusing separation (data not shown).

To detect tyrosine phosphorylation in EGF-stimulated phosphoproteins, we transferred total proteins after electrophoresis to nylon membranes and treated them with alkali. Comparison of Fig. 2A and 2C shows that EGF enhanced the alkali-stable phosphorylation of the two 42-kDa and the two 45-kDa proteins. To determine the nature of the phosphorylated residues in the five proteins whose phosphorylation is stimulated by EGF, we transferred total proteins after elec-

TABLE 3. Proteins whose phosphorylation is modified in response to diverse mitogenic agents

Phosphoproteins (kDa)	Phosphorylation with the following agents ^a :		
	EGF	TPA or diC8	TSH or forskolin
28, two 42, two 45	+	+	0
15, two 34, three 43, 54, two 55, 56, 63, two 65, 68	0	+	0
Two 16, 22, 23, 35, two 37, 50, 59, two 70	0	0	+
16, two 17, 19, 20, 56	0	—	—
15	0	0	—

^a +, Increase in phosphorylation; —, decrease in phosphorylation; 0, no response.

trophoresis to nitrocellulose membranes; the five proteins of interest were then extracted and subjected to phosphoamino acid analysis. As suggested by the disappearance of the phosphate linked to the 28-kDa protein during alkali treatment, this protein contained only phosphoserine (data not shown). Figure 4 shows the phosphoamino acid composition of the pair of 42-kDa proteins. In an attempt to determine the nature of the phosphoamino acids present in the two 42-kDa proteins in the basal state, the exposure time of the autoradiographs was longer for control cells than for EGF-treated cells. The results show that phosphotyrosine and phosphoserine were detected in both 42-kDa proteins (Fig. 4A and 4C) and that additional phosphothreonine was detectable in the more acidic protein (Fig. 4A). To our knowledge, our studies demonstrate for the first time the nature of the phosphoamino acids present in the two 42-kDa proteins before stimulation of the cells. In EGF-treated cells, phosphorylation of tyrosine residues was stimulated in both 42-kDa proteins (Fig. 4B and 4D), while phosphorylation of serine and threonine residues was stimulated only in the more acidic 42-kDa proteins (Fig. 4B). The phosphoamino acid analysis of the two 45-kDa proteins of EGF-treated cells showed that the two 45-kDa proteins contained phosphoserine and phosphothreonine and only trace amounts of phosphotyrosine (data not shown). Thus, five common proteins had their phosphorylation increased by EGF and TPA, and these phosphorylations took place on the same residues.

Kohno (20) has shown that EGF stimulates the phosphorylation of nine proteins in fibroblasts. Five of these proteins show a molecular weight, isoelectric pH, and composition of phosphoamino acids similar to those of the five EGF-stimulated phosphoproteins identified in this work. In our dog thyroid cells, EGF did not increase the phosphorylation of the 36-kDa protein, which is a major tyrosine-phosphorylated protein in transformed cells and in a few types of fibroblasts treated with platelet-derived growth factor. The absence of phosphorylation of this 36-kDa protein was also noticed in several types of EGF-stimulated fibroblasts and suggests that the phosphorylation of this protein is not involved in the mitogenic pathway elicited by EGF (11).

Effect of TSH on protein phosphorylation. Figure 5B presents the phosphorylation pattern obtained when the thyroid cells were incubated with 1 mU of TSH per ml for 15 min. A comparison of Fig. 5A (control cells) and 5B shows that TSH modified the phosphorylation of at least 18 polypeptides (in five of five experiments). The phosphorylation was increased by TSH in 11 proteins (a to k, Table 4) and decreased in 7 others (1' to 7', Table 3). Among these dephosphorylated proteins, six were phosphopeptides whose radioactivity was

FIG. 2. Alkali-resistant phosphoproteins from thyroid cells treated with TPA or EGF. Quiescent dog thyroid cells were labeled with $^{32}\text{PO}_4$ for 8 h, and TPA or EGF was added 15 min before the end of the labeling period. Phosphoproteins were transferred after electrophoresis to nylon membranes and treated with alkali as described in Materials and Methods. (A) No addition; (B) TPA, 50 ng/ml; (C) EGF, 50 ng/ml. The proteins whose phosphorylation was modified are indicated by arrowheads.

FIG. 3. Phosphoamino acid analysis of the two 42-kDa proteins from thyroid cells treated with TPA. Quiescent dog thyroid cells were labeled with 3 mCi of $^{32}\text{PO}_4$ per ml for 8 h, and TPA was added 15 min before the end of the labeling period. Phosphoamino acid analysis was performed as described in Materials and Methods. Marker phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were added to the sample and located by ninhydrin staining. The broken lines indicate the position of the ninhydrin-colored spot on control plates or on plates on which the radioactive spot is absent. The phosphoamino acids of the 42-kDa proteins were detected by autoradiography. (A and B) The acidic form of the 42-kDa proteins; (C and D) the basic form of the protein. The Cerenkov counts per minute applied and exposure times were as follows: (A) control cells, 72 cpm, 10 days; (B) TPA-treated cells, 490 cpm, 10 days; (C) control cells, 50 cpm, 24 days; (D) TPA-treated cells, 250 cpm, 24 days.

also decreased by TPA (Table 3). The proteins whose phosphorylation was stimulated by TSH were different from those whose phosphorylation was enhanced by TPA or EGF (Table 3). Increases in the phosphorylation state of these 11 proteins were confirmed by excision of the proteins from the gel and determination of their radioactivity (Table 4). No stimulation of the incorporation of $^{32}\text{PO}_4$ in the two 42-kDa proteins could be detected after TSH treatment of the cells. Since it has been shown that the effect of TSH on thyroid cell proliferation is reproduced by agents that increase the intracellular concentration of cyclic AMP (34), we studied the effect of forskolin on protein phosphorylation in these cells. Forskolin (50 μM) exactly reproduced the effect of TSH on thyrocyte protein phosphorylation (Table 3). Using one-dimensional gel electrophoresis of dog thyroid proteins, it has been shown that TSH stimulates the phosphorylation of three proteins of 17, 33, and 38 kDa (18, 40), but their relationship to the proteins phosphorylated in response to TSH in this study is difficult to determine.

To investigate the nature of the phosphorylated residues in the proteins whose radioactivity is increased by TSH and to confirm the absence of stimulation by TSH of phosphorylation of the tyrosine residues of the two 42-kDa phosphoproteins, the proteins extracted from TSH-treated thyroid cells or from control cells were transferred after electrophoresis to nylon membranes and treated with alkali. The phosphate linked to 10 of the TSH-phosphorylated proteins did not resist alkali treatment. Protein g was alkali stable, but the phosphoamino acid analysis of this protein revealed only phosphoserine (data not shown). Moreover, no additional changes in the phosphorylation state of the proteins were

FIG. 4. Phosphoamino acid analysis of the two 42-kDa proteins from thyroid cells treated with EGF. Quiescent dog thyroid cells were labeled with 3 mCi of $^{32}\text{PO}_4$ per ml for 8 h, and EGF (50 ng/ml) was added 15 min before the end of the labeling period. Phosphoamino acid analysis was performed as described in the legend to Fig. 3. (A and B) The acidic form of the 42-kDa proteins; (C and D) the basic form. The Cerenkov counts per minute applied and exposure times were as follows: (A) control cells, 350 cpm, 21 days; (B) EGF-treated cells, 778 cpm, 12 days; (C) control cells, 340 cpm, 21 days; (D) EGF-treated cells, 515 cpm, 12 days. Y, T, and S are defined in the legend to Fig. 3.

observed after alkali treatment of the nylon membranes, showing an absence of tyrosine phosphorylation induced by TSH.

Kinetics of protein phosphorylation in response to EGF and TSH. As the phosphorylation of the two 42-kDa proteins was not modified after 15 min of TSH action, we investigated the kinetics of TSH action during the whole length of the prereplicative phase, from 0 to 19 h. Most of the proteins whose phosphorylation was modified after 15 min of TSH action (Fig. 5) were already affected after 5 min of its action. No additional TSH-phosphorylated proteins were detected

TABLE 4. Characteristics of the proteins whose phosphorylation is stimulated in response to TSH or forskolin

Reference letter ^a	$10^3 M_r$	^{32}P labeling of proteins (cpm) ^b		
		-TSH	+TSH	Fold increase
a	70	ND ^c	43 $\pm$ 17	
b	70	ND	33 $\pm$ 7	
c	59	63 $\pm$ 15	179 $\pm$ 17	2.8
d	50	ND	412 $\pm$ 24	
e	37	167 $\pm$ 2	997 $\pm$ 23	6.0
f	37	85 $\pm$ 11	205 $\pm$ 30	2.4
g	35	ND	53 $\pm$ 13	
h	23	ND	94 $\pm$ 9	
i	22	ND	55 $\pm$ 16	
j	16	ND	48 $\pm$ 12	
k	16	76 $\pm$ 21	160 $\pm$ 20	2.1

^a Reference letters identify the proteins shown in Fig. 5.

^b Cells were labeled with $^{32}\text{PO}_4$ for 8 h, and 1 mU of TSH per ml was added 15 min before the end of the culture period. The experimental details are described in Table 2, footnote b. Values are expressed as mean $\pm$ range of duplicate assays. The background value of radioactivity was 40 $\pm$ 4 cpm.

^c ND, Not detected.

FIG. 5. Two-dimensional gel analysis of phosphoproteins from thyroid cells treated with TSH. Quiescent dog thyroid cells were labeled with $^{32}\text{PO}_4$ for 8 h, and TSH was added 15 min before the end of the labeling period. Samples were subjected to electrophoresis in two dimensions as described in Materials and Methods. (A) No addition; (B) TSH, 1 mU/ml. The proteins whose phosphorylation was increased by TSH are identified with an arbitrary letter (a to k) in panel B. The proteins whose phosphorylation was decreased in the presence of TSH are identified with an arbitrary number (1' to 7') in panel A.

at any later time point of TSH action. After 7 h of TSH action, the phosphorylation of four of these target proteins disappeared, while the increased phosphorylation of five others declined but remained higher than in control cells (data not shown).

Since no stimulation by TSH of the phosphorylation of the two 42-kDa proteins was detected in two-dimensional gels, the effect of TSH on protein phosphorylation was also studied after alkali treatment of the phosphoproteins. Figure 6 shows the kinetics of alkali-resistant phosphorylation of the two 42-kDa proteins after either EGF or TSH treatment of the same thyroid cell culture 5 min, 15 min, 1 h, and 4 h before the end of the culture period. Stimulation of the 42-kDa protein phosphorylation was detectable after 5 min of EGF action and was still detectable after 4 h of EGF action. In the TSH-treated cells, the level of phosphorylation of the 42-kDa proteins was indistinguishable from the control level at the different time points studied. The same results were obtained when the cells were treated for longer periods with TSH (from 4 to 19 h) (Fig. 7).

DISCUSSION

Protein phosphorylation, whether by the receptor itself or by kinases modulated by intracellular signals such as cyclic AMP, diacylglycerol, or calmodulin, is commonly accepted as an obligatory step in the pathways of mitogenic activation. We have shown that a differentiated epithelial cell, the

dog thyrocyte, is induced to proliferate in response to three different signals: TSH acting through cyclic AMP, EGF, and TPA (33–35). In this work, we analyzed the protein phosphorylation response characterizing, in this cell, these mitogenic pathways.

In dog thyroid cells as in fibroblasts (19, 22), EGF did not seem to stimulate the turnover of phosphatidylinositol; it had no effect on inositol phosphate accumulation (IP_3 , IP_2 , or IP_1), it did not increase intracellular free Ca^{2+} concentration measured by the quin $\{2\text{-}[(2\text{-bis}[\text{carboxymethyl}]\text{amino-5-methyl-phenoxymethyl}]-6\text{-methoxy-8-bis}[\text{carboxymethyl}]\text{aminoquinoline})\}$ technique, and it did not affect the Ca^{2+} efflux of cells loaded with $^{45}\text{Ca}^{2+}$ (E. Laurent and E. Raspé, unpublished data). Our data showed that stimulation of thyroid cells with EGF increases the phosphorylation of five polypeptides, including two 42-kDa proteins phosphorylated on tyrosine, threonine, and/or serine residues. The molecular weight, isoelectric pH, phosphorylated amino acid composition, and low abundance of these two 42-kDa proteins suggest that they are identical to the two 42-kDa proteins described in fibroblasts whose phosphorylation on tyrosine is believed to represent a common response of cells to mitogens (9, 13, 16, 20, 21, 27).

TPA stimulated the phosphorylation of 19 polypeptides in our system. This effect was entirely mimicked by diC8, a diacylglycerol analog, which strongly suggests that protein phosphorylation by TPA implicates protein kinase C. Five common proteins were phosphorylated with the same resi-

FIG. 6. Radioautographic detail of alkali-resistant 42-kDa phosphoproteins from thyroid cells. A characteristic section of the two-dimensional pattern of proteins (corresponding to a molecular weight range of 50 to 34×10^3) was chosen to illustrate the effect of EGF (50 ng/ml) and TSH (1 mU/ml) on the phosphorylation of the two 42-kDa proteins. Quiescent dog thyroid cells were labeled for 20 h with $^{32}\text{PO}_4$, and EGF or TSH was added at different time points before the end of the labeling period as indicated. Phosphoproteins were transferred after electrophoresis to nylon membranes and submitted to alkali treatment as described in Materials and Methods. Arrowheads indicate the positions of the two 42-kDa proteins.

due specificity in response to both EGF and TPA. Included in these five common phosphoproteins were the two 42-kDa proteins phosphorylated on tyrosine, threonine, and/or serine residues. Since the presumed receptor of TPA, protein kinase C, is specific for serine and threonine phosphorylation, tyrosine phosphorylation implies that protein kinase C modulates the activity of a protein tyrosine kinase or a protein phosphotyrosine phosphatase. The EGF receptor is not implicated as it has been shown that its tyrosine protein kinase activity is inhibited by TPA (3). This is the first time that the phosphorylation of these 42-kDa proteins in response to mitogens acting through the receptor tyrosine kinase pathway or through the protein kinase C pathway has been demonstrated in primary cultures of differentiated

FIG. 7. Radioautographic detail of alkali-resistant 42-kDa phosphoproteins from thyroid cells. A characteristic section of the two-dimensional pattern of proteins (corresponding to the molecular weight range of 50 to 34×10^3) was chosen to illustrate the effects of TSH on the phosphorylation of the two 42-kDa proteins during the whole length of the prereplicative phase. Quiescent dog thyroid cells were labeled for 20 h with $^{32}\text{PO}_4$, and TSH (1 mU/ml) was added at different time points before the end of the labeling period as indicated. Phosphoproteins were transferred after electrophoresis to nylon membranes and submitted to alkali treatment as described in Materials and Methods. Arrowheads indicate the positions of the two 42-kDa proteins.

epithelial cells. The fact that EGF and TPA share, at least in part, a common response to protein phosphorylation is not surprising. TPA mimics all the effects of EGF in dog thyroid cells; both EGF and TPA induce proliferation and repress differentiation expression as evaluated by iodide trapping and thyroglobulin gene expression. In addition, the long-term morphological changes induced by EGF and TPA are similar (33). It should also be noted that EGF and TPA enhance the accumulation of the proto-oncogene *c-myc* mRNA after 3 and 6 h of action (31) and that they specifically stimulate the synthesis of one protein (*Mr* 81,000) during the prereplicative phase (23; unpublished data). These data

therefore suggest that the EGF and TPA mitogenic pathways join or at least overlap at some point(s) beyond the initial signal receptor interaction. On the other hand, TPA induces the phosphorylation of many more proteins than EGF. The greater number of proteins phosphorylated by TPA in dog thyroid cells may be linked to the fact that while EGF has no rapid effect on these cells, TPA acutely alters their morphology and iodine metabolism (26, 33, 42).

TSH induced the phosphorylation of 11 specific polypeptides in dog thyrocytes. It is known that most of the effects of TSH in the dog thyroid, whether acute, such as the activation of thyroid hormone synthesis and secretion, or delayed, such as the induction of proliferation and differentiation expression, are mediated by cyclic AMP (15, 34, 37). It is therefore not surprising that forskolin, a general non-specific activator of adenylate cyclase, induces the phosphorylation or dephosphorylation of the same proteins as TSH. It is rather striking that no common protein phosphorylation is induced by TSH and cyclic AMP on the one hand and by EGF and TPA on the other hand. The absence of a rapid phosphorylation of the 42-kDa protein in TSH-treated cells which are stimulated to proliferate as well as EGF- or TPA-treated cells suggests that this phosphorylation is not a common obligatory step in the pathway to mitogenesis. Until now, the rapid phosphorylation of these two 42-kDa proteins has been observed in fibroblasts from different species induced to proliferate by growth factors which elicit the receptor tyrosine kinase pathway or the inositol phosphate pathway or both and by growth factors whose mode of action is still unknown (multiplication-stimulating activity; trypsin; serum), but it has not been investigated in the cyclic AMP proliferative pathway. To answer the question whether the kinetics of TSH action on the 42-kDa protein phosphorylation could be different from those of EGF and TPA, we examined the effect of TSH on protein phosphorylation during the whole length of the prereplicative phase of the cell cycle from 0 to 19 h. No alkali-resistant modifications induced by TSH at different time points of the prereplicative phase of the cell cycle were observed, and in particular, no stimulation of the phosphorylation of the two 42-kDa proteins was detected.

Our results also showed that TSH decreases the phosphorylation of seven polypeptides. The phosphorylation of six of these polypeptides was decreased both by TSH and TPA. No dephosphorylation of proteins phosphorylated in the basal state was detected after EGF treatment. This common pattern of dephosphorylation of phosphoproteins after TSH or TPA action might be associated with the rapid and important changes in cell morphology and iodine metabolism induced by these agents (26, 32, 33, 42). The identity of these seven polypeptides is presently unknown, although the two proteins of 19 and 20 kDa could be similar to the 19- and 20-kDa proteins shown to be dephosphorylated in another laboratory after TSH or TPA treatment of dog thyroid cells and suggested to be the myosin light chains (17, 18).

In conclusion, within the limitations of the two-dimensional gel electrophoresis technique (10), we observed no overlap in the patterns of protein phosphorylation stimulated by TSH or forskolin and those stimulated by EGF or TPA. Thus, we did not identify one common protein whose phosphorylation is induced by each of the different mitogenic stimuli. However, a common mitogen-stimulated phosphorylation of a protein present at very low concentration and of which only a small proportion is phosphorylated could have been missed. On the other hand, we showed that EGF and TPA share, at least in part, a common pattern of

protein phosphorylation: they stimulate the phosphorylation of the same five polypeptides with identical phosphoamino acid specificities. Another major finding in this study was that TSH phosphorylates a distinct set of intracellular proteins which does not involve tyrosine phosphorylation of the two 42-kDa proteins but rather implicates cyclic AMP and activation of the cyclic AMP-dependent kinases by this nucleotide. Several other arguments are in favor of the independence of the mitogenic pathways elicited by EGF and TPA on the one hand and by TSH and forskolin on the other hand (33, 35, 36). (i) While EGF and TPA have no additive effects, EGF synergizes with forskolin and dibutyryl cyclic AMP to induce DNA synthesis. (ii) Forskolin controls the progression in the prereplicative phase until a late commitment point. Kinetic experiments suggest that prereplicative events induced by EGF or forskolin are not interchangeable, in such a way that when EGF and forskolin are not added together, events induced by the second factor can no longer be integrated during the prereplicative phase set by the first factor. (iii) Low concentrations of insulin are sufficient to support the mitogenic effect of TSH, whereas higher concentrations that could activate insulinlike growth factor receptors are necessary to obtain the mitogenic effect of EGF. (iv) The pattern of protein synthesis induced by these two classes of agents during the prereplicative phase is different (23).

The only overlap in TSH and EGF or TPA action is the induction of *c-myc* mRNA (31). However, in other systems the expression of *c-myc* is insufficient to induce mitogenesis. Moreover, in the dog thyrocytes, the effects of EGF and TPA on this induction are long lasting and stronger than those of TSH and forskolin, although their mitogenic effects are weaker (31). The data obtained on the proliferation of the dog thyrocyte thus indicate that besides the already clearly delineated but early converging mitogenic pathways involving receptor tyrosine kinase activation and hydrolysis of inositol lipids, another largely independent mitogenic pathway should be considered: the cyclic AMP cascade.

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