Phosphorylation of Mitogen-activated Protein Kinases Is Involved in the Epidermal Growth Factor and Phorbol Ester, but Not in the Thyrotropin/cAMP, Thyroid Mitogenic Pathway*

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In dog thyroid epithelial cells (thyrocytes) in primary culture, thyrotropin (TSH) acting through cAMP induces proliferation and differentiation expression, whereas epidermal growth factor (EGF) and tumor-promoting phorbol esters induce proliferation and dedifferentiation. In these cells we have demonstrated mitogen-activated protein (MAP) kinase phosphorylation by 32P labeling and two-dimensional gel electrophoresis and by immunodetection with anti-MAP kinase antibody and anti-phosphotyrosine antibodies after one- or two-dimensional gel electrophoresis. MAP kinase localization was demonstrated by immunochemical staining. We show the following results. (i) As in other systems, EGF and phorbol esters induced p42 and p44 MAP kinases phosphorylation on tyrosine, serine, and threonine. This effect was rapid, peaking after 5 and 15 min, respectively, followed by a slow decline thereafter. It preceded a translocation of MAP kinase immunoreactivity from cytoplasm to nucleus. (ii) Carbachol, a potent stimulator of the Ca++-phosphatidylinositol cascade which is unable to induce DNA synthesis, stimulated MAP kinases phosphorylation and nuclear staining with kinetics similar to those observed after EGF action, indicating that MAP kinase phosphorylation was not sufficient for mitogenesis. (iii) The cAMP-dependent mitogenic cascade elicited by TSH and forskolin did not involve the phosphorylation and nuclear translocation of p42 and p44 MAP kinases at any time during the entire prereplicative phase. Activation of MAP kinases by phosphorylation is therefore not a necessary step in the G0-G1 transition in this mitogenic cascade.

In the receptor-tyrosine kinase mitogenic cascades, interaction of the growth factor with its receptor activates its catalytic intracellular carboxyl-terminal domain leading to its own phosphorylation on specific tyrosine residues and to its association with various first target proteins through specific src homology 2 domains (1). This is followed by activation of p21v (2) and stimulation of mitogen-activated protein (MAP) kinase through phosphorylation on both threonine and tyrosine (3). MAP kinase is similarly phosphorylated in the throbmin and phorbol ester-protein kinase C pathways (4, 5). MAP kinase has been shown to phosphorylate a whole set of proteins involved in the development of the mitogenic response including S6 kinase II (6), the products of oncoproteins c-myc (7) and c-jun (8), and the transcription factor p62CRE (9). These results suggested that this activating phosphorylation is a common step of all mitogenic cascades (10, 11). The dog thyroid cell is a prototype of cells in which the cAMP cascade is mitogenic (12–14). In this report we show that in these cells the activating phosphorylation of MAP kinase is a common step of both phorbol ester and growth factor cascades but that it is not involved in the mitogenic cAMP pathway.

MATERIALS AND METHODS

Primary Cultures—Dog thyrocytes were cultured in monolayer (2 × 10^4 cells/cm²) in the following medium: Dulbecco’s modified Eagle’s medium + Ham’s F-12 medium + MCDB104 medium (2:1, v/v) supplemented by 5 µg/ml insulin, 40 µg/ml ascorbic acid, and antibiotics (15). After 4 days of culture, the cells were incubated for a 48-h period with the mitogenic agents and with [3H]thymidine (10 µCi/ml, 3 × 10^5 M) and deoxycytidine (10^-5 M) for the last 24 h (12). The number of cells entering into DNA synthesis was estimated by the frequency of the [3H]thymidine-labeled nuclei as revealed by autoradiography. In each duplicate dish at least 1000 nuclei chosen at random in different fields were counted in blind (12). For 32P radioactive labeling, after 4 days of culture, the cells were incubated for 8 h in the labeling medium: Eagle minimal essential medium containing 9 µM KH2PO4, 5 µg/ml insulin, and 1 mCi/ml 32PO4 (carrier-free, Amersham, United Kingdom).

Gel Electrophoresis—At the end of the culture, the cells were lysed and the proteins solubilized in 200 µl of lysis buffer, composed for sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (one-dimensional gels) (0.06 M Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol) and for two-dimensional gels (3.6 M urea, 2% w/v) Nonidet P-40, 2% carrier ampholyte, pH 7–9, 100 mM dithiothreitol) (16). For two-dimensional gel electrophoresis, proteins were first separated by isoelectric focusing, on cylindrical gels with 3.2% Servalyte, pH 5–7, plus 0.8% Servalyte, pH 2–11. For two-dimensional and one-dimensional gel electrophoresis, proteins were separated according to molecular mass on sodium dodecyl sulfate polyacrylamide gel slab gels (total length of the separation gel = 18 cm). Proteins were transferred to a nitrocellulose membrane, pH 79 (Schleicher and Schüll, Dassel, Germany) for 16 h at 60 V and 4 °C as described (16). Polyclonal antibody against a COOH-terminal peptide (15 amino acids) of Xenopus MAP kinase (1913.2) (17) was used at a 1/2000 dilution, and 125I-protein A (Amerham, 5 × 106 dpm/ml) was used as a secondary reagent for develop-
opment by autoradiography. Mouse monoclonal antibody against phosphotyrosine PY20 (ICN, Costa Mesa, CA) was used at a 1/1000 dilution with 3H-labeled anti-mouse Ig whole antibody from sheep (Amersham, United Kingdom) as secondary reagent.

**Indirect Immunofluorescence—**Cells in Petri dishes (2 x 10^6 cells/cm^2) were fixed with 1% paraformaldehyde for 90 s at 4°C and then with methanol for 10 min at −20°C, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 5% horse serum for 30 min. Cells were then incubated overnight at 4°C with the 1913.2 polyclonal antibody against MAP kinases at 1/1000 dilution in PBS containing 0.1% bovine serum albumin (PBS-BSA), washed with PBS-BSA, and incubated for 2 h at room temperature with fluorescent-conjugated donkey anti-rabbit immunoglobulins (Amersham) at 1/50 dilution in PBS-BSA. Cells were viewed with Leitz epifluorescence microscope (50× oil immersion lens). Microphotographs were taken using 400 iso Fujichrome films with a fixed 1-min exposure.

**RESULTS**

After 4 days without mitogenic agents (control medium) dog thyrocytes were spread and quiescent. Addition of TPA, EGF, TSH, forskolin, but not carbamylcholine, induced, after a similar prereplicative phase of about 20 h, a progressive accumulation of [3H]thymidine-labeled nuclei (12, 15, 18, 19) (Table I).

Immunodetection by anti-MAP kinase antibody shows that thyroid cells contain two MAP kinase-related proteins (p42 and p44) and that these proteins, as indicated by a reduced mobility in SDS gels (17, 20), are phosphorylated in response to a 5-min stimulation of the cells by EGF and TPA but not by TSH (Figs. 1A, 2, and 3). Prior incubation of the antibody 1913.2 with the immunogen peptide suppressed only the 42-

**TABLE I**

**Effect of mitogen treatment on [3H]thymidine incorporation into the nuclei**

The different agents were added to the culture medium after 4 days of culture for 48 h. [3H]Thymidine was present for the last 4 h of culture for each condition, which was always tested in duplicate dishes.

| n  | Treatment          | [3H]Thymidine-labeled nuclei (%) |
|----|--------------------|---------------------------------|
| 12 | Control            | 1.3 ± 0.4                       |
| 9  | TSH (1 milliunit/ml) | 40 ± 4.5                        |
| 3  | Forskolin (2 x 10^{-5} M) | 41 ± 3                          |
| 6  | EGF (50 ng/ml)     | 22 ± 6                          |
| 6  | TPA (100 ng/ml)    | 35 ± 6                          |
| 5  | Carbamylcholine (10^{-3} M) | 2.5 ± 1.4                      |

**FIG. 1. Effect of TSH, EGF, and TPA on MAP kinase phosphorylation.** Anti-MAP kinase immuno blot (A) or anti-phosphotyrosine (PY20) immuno blot (B) of whole cell extracts from dog thyrocytes stimulated for 5 min with no agent (lane 1), 1 milliunit/ml TSH (lane 2), 100 ng/ml EGF (lane 3), or 100 ng/ml TPA (lane 4) after 4 days of culture. Proteins (100 µg) were loaded in each lane. Upper panel of A (from 44 to 200 kDa) has been exposed twice as long as lower panel (from 30 to 42 kDa).

**FIG. 2. Time course of MAP kinases phosphorylation after stimulation by TSH, EGF, and TPA.** Anti-MAP kinase immunoblot of whole cell extracts from dog thyrocytes stimulated for the times indicated on the figure with the following: lane 1, no agent; lane 2, 1 milliunit/ml TSH; lane 3, 100 ng/ml EGF; lane 4, 100 ng/ml TPA; lane 5, 2 x 10^{-5} M forskolin. Proteins (50 µg) were loaded in each lane. Arrows indicate the position of the non-phosphorylated forms of p42 and p44. A short exposure time was chosen to allow maximal resolution of the two forms of p42 MAP kinase.

**FIG. 3. Time course of MAP kinases phosphorylation after stimulation by carbamylcholine, TPA, and EGF.** Anti-MAP kinase immunoblot of whole cell extracts from dog thyrocytes stimulated for the times indicated on the figure with the following: lane 1, no agent; lane 2, 1 milliunit/ml TSH; lane 3, 100 ng/ml EGF; lane 4, 100 ng/ml TPA; lane 5, 2 x 10^{-5} M forskolin. Proteins (50 µg) were loaded in each lane. Arrows indicate the position of the non-phosphorylated forms of MAP kinases. For a longer exposure time see Fig. 3.

and 44-kDa bands (not shown). Treatment of the cells with EGF or TPA but not with TSH was followed by the appearance of two bands of 42 and 44 kDa as revealed by anti-phosphotyrosine antibody (PY20) in Western blots, which suggests that this phosphorylation involves tyrosine residues (Fig. 1B).

The kinetics of MAP kinases phosphorylation were then estimated by immunodetection by anti-MAP kinase antibody after a treatment of the cells with the different agents (Figs. 2 and 3). Stimulation of 42- and 44-kDa MAP kinase phosphorylation was maximal after 5 min of EGF action and after 15 min of TPA action and persisted, in both cases, for at least 8 h. Carbamylcholine had an effect similar to that of EGF on the phosphorylation of both kinases. In the TSH- and forskolin-treated cells, the level of phosphorylation of the MAP kinases was indistinguishable from the control level (Figs. 2 and 3).

Two-dimensional gel electrophoresis and immunodetection by anti-MAP kinase antibody show that treatment of the cells with EGF, TPA (Fig. 4), or 10^{-5} M carbamylcholine (not
MAP Kinase Is Not Phosphorylated in cAMP-induced Mitogenesis

The different stimulating agents on the phosphorylation of MAP kinases extracted from cells incubated with [32P]phosphate proteins, of two new isoforms (B and A) characterized by p44 present in two-dimensional gels after separation of total kinases. Dog thyrocytes were exposed for 15 min to TSH (1 milliunit/ml), EGF (100 ng/ml), or TPA (100 ng/ml) after 4 days of culture.

The staining was essentially cytoplasmic, but a weak acidic isoform of both proteins (form A), whereas form B contained mostly phosphotyrosine (21). In some experiments the intensity of form B was very weak. In Swiss 3T3 fibroblasts, only form A corresponded to the activated MAP kinase (22, 23).

Unstimulated dog thyrocytes after a 4-day culture in control medium displayed a marked MAP kinase immunoreactivity, which was homogeneously distributed within the cell population.

The staining was essentially cytoplasmic, but a weak nuclear immunoreactivity was also evident (Fig. 5A). Two hours after stimulation with EGF (Fig. 5B), carbamylcholine (Fig. 5C), and most markedly with TPA (Fig. 5D), an increase of nucleoplasm staining with a clear exclusion of nucleoli and a concomitant decrease in the cytoplasm labeling were observed in many cells. The effect was observed only in a fraction of EGF or carbamylcholine-stimulated cells but in the great majority of TPA-treated cells (Fig. 5).

The increase of nuclear MAP kinase immunoreactivity was maximal after 2 h of stimulation, and detectable after 1 h but not after 20 min, suggesting that it was delayed compared to the phosphorylation of these proteins (not shown). It was also more transient in EGF and carbamylcholine-stimulated cells, whereas it was still observed after 8 h of stimulation by TPA (not shown). TSH did not produce any effect on the nuclear localization of MAP kinases in cells that presented the characteristic morphological response to this hormone (Fig. 5E), at any of the time points investigated (from 5 min to 16 h).

Preabsorption of the MAP kinase antibody with the immunogen peptide blocked almost completely the cytoplasmic staining of control cells (Fig. 5F) and nuclear staining of TPA-treated cells (Fig. 5G).

**DISCUSSION**

Dog thyroid epithelial cells in primary culture provide a unique opportunity to compare biochemical events associated with the prereplicative development induced by mitogens as different as TSH, EGF, and TPA. In this system, EGF does not increase inositol phosphate accumulation and intracellular calcium concentration (19). TSH, at mitogenic concentrations that are not superior to 1 milliunit/ml, has no effect on phosphoinositide metabolism and calcium mobilization (19); it exerts its mitogenic as well as its functional effects solely through adenylate cyclase activation, increase in intracellular cAMP, and cAMP-dependent protein kinase stimulation (14).

In contrast, neither EGF nor phorbol esters enhance cAMP accumulation in dog thyroid cells. The three cascades are therefore fully distinct at the level of their primary intracellular signal and/or of the first signal-activated protein kinase.

In this work, we show that in thyroid epithelial cells as in other types of cells, EGF and phorbol esters enhance MAP kinase phosphorylation on tyrosine, threonine, and serine early in the respective mitogenic pathways and that this phosphorylation only slowly decline thereafter. Although in other systems, this phosphorylation has been shown to result from an activation of MAP kinase kinase, in this work we only study the state of phosphorylation of the enzyme that might be altered either at the phosphorylation or at the dephosphorylation steps. These effects preceded a translocation of MAP kinases from cytoplasm toward nucleus. The MAP kinase species involved in this translocation is not known, since both p42 and p44 forms are recognized by our MAP kinase antibody. This resembles the transfer observed in serum-stimulated HeLa cells (24), but was more delayed here. It might be determinant in the effects of MAP kinases in the EGF and TPA mitogenic pathways, since part of their action could be through phosphorylation of nuclear transcription factors including c-Jun (8), c-Myc (7), and p62MyC (9). It could also be involved in the dedifferentiation induced by these agents (14). The intercellular heterogeneity in the nuclear translocation of MAP kinases is interesting, since it...
could contribute to explain the intercellular heterogeneity of DNA synthesis responses to EGF and TPA (15, 18).

As in some other systems (6, 25), MAP kinase activation is not sufficient for mitogenesis. Carbamylcholine, which is the most potent trigger of the dog thyrocyte Ca\(^{2+}\)-phosphatidylinositol cascade but which is unable to induce DNA synthesis in these cells (19), strongly stimulated the phosphorylation of MAP kinases with kinetics similar to those observed after EGF action. Like EGF, it induced an heterogeneous increase in nuclear staining of MAP kinases. Carbamylcholine also elicits the accumulation of c-fos and c-myc mRNA with a time course characteristic of the EGF and protein kinase C pathways (19). All these "early mitogenic events" are therefore not sufficient by themselves to commit the entry of the dog thyrocytes into S phase.

In the present study, we demonstrate that the CAMP-dependent mitogenic cascade elicited by TSH and forskolin in dog thyroid cells does not involve the phosphorylation and nuclear translocation of p42/p44 MAP kinases, in sharp contrast with prominent positive effects of EGF, TPA, and carbamylcholine. This is at variance with the current hypothesis (10, 11) that activation of MAP kinases by phosphorylation constitutes a necessary integrator step in the G\(_0\)/G\(_1\) transition, involved in nuclear transduction of the external signals. The CAMP-dependent mitogenic pathway of thyrocytes thus differs from CAMP-independent pathways elicited by mitogenic factors as diverse as those acting on tyrosine kinase receptors, G\(_\text{i}\) and G\(_\text{q}\)-coupled receptors or protein kinase C, and various oncogene products including Src, Raf, Ras, and Gip2, which all converge early on MAP kinase phosphorylation and activation (5, 6, 26).

Thus as in other aspects, the CAMP mitogenic cascade in thyrocytes contradicts generalizations on the necessary biochemical steps of mitogenesis. In contrast to what is observed in the CAMP-independent proliferation cascades, CAMP uncouples the expression of c-Fos and c-Jun components of the AP-1 factor as it down-regulates c-jun mRNA (14). After its initial induction by CAMP, c-myc mRNA accumulation is also rapidly down-regulated by a CAMP-dependent mechanism (14). Such discrepancies, both on the induction of these transcription factors and on the activation of MAP kinases that regulate their activity, could explain late differences in the synthesis of proteins during G\(_1\) phase (27) and the fact that the CAMP cascade induces both proliferation and differentiation expression whereas the other mitogenic pathways deifferentiate the thyroid cells (14). The parallelism rather than the convergence of the different mitogenic pathways thus suggests that the final decision at START of the beginning of DNA synthesis can result from the action of different combinations of signals on a supposedly common trigger.

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