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Characterization and Identification as Cofilin and Destrin of Two Thyrotropin- and Phorbol Ester-Regulated Phosphoproteins in Thyroid Cells

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Using separation of total cellular proteins by two dimensional (2-D) gel electrophoresis (isoelectric focusing/SDS–PAGE) we have characterized two regulated proteins, p21 and p19, in dog thyroid cells. We have used the same 2-D gel technique to purify these proteins before their trypsin cleavage and partial sequencing. Three peptides were sequenced in the case of p19 and two peptides in the case of p21. The Swiss-Prot protein sequence database revealed that p19 was identical to destrin/ADF (actin depolymerizing factor) and p21 to cofilin, two closely related and widely distributed actin-binding proteins. This was further verified by cross-reactivity with specific antibodies against brain cofilin and chicken ADF. We have demonstrated, using 2-D gel electrophoresis with a nonequilibrium pH gradient in the first dimension (nonequilibrium pH gradient electrophoresis/SDS–PAGE) that, in the thyroid cell, cofilin and destrin/ADF were present, under control conditions, in two forms: a phosphorylated and an unphosphorylated one. Thyrotropin (TSH), through cyclic AMP, provoked a very rapid dephosphorylation of these two proteins, which was already maximal after 20 min of action, whereas their dephosphorylation in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was slower. This suggests that dephosphorylation of cofilin and destrin/ADF by TSH could be implicated in the disruption of actin-containing stress fibers and in the reorganization of microfilaments induced by this hormone. Epidermal growth factor, which does not induce acute morphological changes in thyroid cells, did not affect the state of phosphorylation of cofilin and destrin/ADF except for a delayed decrease (after 24 h) of destrin/ADF phosphorylation. A 10% dimethyl sulfoxide treatment of thyroid cells also induced rapid dephosphorylation of destrin and cofilin. This was accompanied by a reorganization of actin microfilaments that clearly resembles the one induced by TSH and by the appearance of intranuclear cofilin-containing rods. However, these rod structures were not observed in response to TSH, forskolin, or TPA, suggesting that dephosphorylation of cofilin correlates with the reorganization of actin microfilaments but not with the nuclear transport of cofilin. We propose that the dephosphorylation of destrin and cofilin could be involved in the TSH-stimulated macropinocytic activity, a key process in thyroid hormone secretion.

INTRODUCTION

For several years we have used two-dimensional (2-D) polyacrylamide gel electrophoresis [IEF (isoelectric focusing)/SDS–PAGE] to identify regulated proteins in dog thyroid cells [1]. After elution from the 2-D gels, these proteins of interest can be partially sequenced for a search of identity using protein databases or for the generation of specific DNA probes for cloning purposes.

In vivo, thyrotropin (TSH) is the main element which controls thyroid function and growth [2]. In the dog thyroid cell, TSH exerts all of its effects via a receptor coupled to the adenylate cyclase–cyclic AMP–protein kinase system. The function of the thyroid follicular cell is to synthesize and secrete the thyroid hormones triiodothyronine and thyroxine. The synthesis of thyroid hormones involves the thyroperoxidase catalyzed iodination and coupling of specific tyrosine residues in thyroglobulin, which serves as the biosynthetic precursor of the thyroid hormones. The secretion of these hormones in response to TSH involves macropinocytosis of the luminal iodinated thyroglobulin, its hydrolysis in secondary lysosomes, and the release of the hormones [3]. This process involves the formation of pseudopods, which depends on a redistribution of microfilaments [4]. In dog thyroid cells in primary culture TSH, through protein kinase A, and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), through protein kinase C, but not epidermal growth factor (EGF) provoke acute but distinct changes in cell morphology involving a rapid disruption of the actin-containing stress fiber

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network which last for several hours [5]. TSH, but not TPA, also acutely induces phagocytosis by cultured dog thyroid cells [6]. Dog thyroid cells can proliferate in defined medium in response to TSH but also to EGF and to TPA [7, 8, 9]. The cyclic AMP-dependent mitogenic pathway induced by TSH appears essentially distinct from the rapidly converging EGF and TPA cascades as judged from the patterns of protein phosphorylation and synthesis [10, 11, 12, 13]. These distinct mitogenic pathways are associated with opposite effects on the expression of thyroid differentiation [14]: TSH acting through cyclic AMP induces thyroglobulin and thyroperoxidase mRNA accumulation and iodide transport; EGF and TPA antagonize these effects [8, 15, 16].

The aim of this work was to further characterize and to identify, using 2-D gel electrophoresis, two thyroid-regulated proteins, p21 and p19. We have already shown, using this technique (IEF/SDS–PAGE), that the incorporation of [^{35}S]methionine into these proteins was decreased after 6 h exposure of these cells to TSH or to agents which increase the intracellular concentration of cyclic AMP [17] and to TPA (Lamy, unpublished results). At this time point, TSH and TPA affected the incorporation of [^{35}S]methionine into very few other proteins [17; Lamy, unpublished data]. Moreover p19 was the only protein in which [^{35}S]methionine incorporation was decreased in response to a prolonged (24 h) action of all mitogens tested [12, 13].

In this work, we have purified dog thyroid proteins p21 and p19 and identified p21 as cofilin and p19 as destrin/ADF (actin depolymerizing factor). Destrin and cofilin are two widely distributed actin binding proteins with similar amino acid sequences (71% identical) but whose function, *in vitro,* has been reported to be distinct [18, 19, 20, 21]. The interaction of cofilin with actin is sensitive to pH, whereas that of destrin/ADF is not. At near neutral pH, cofilin binds to F-actin in a 1:1 molar ratio of cofilin to actin molecule in the filament, shortens the average length of the filament, and increases the steady-state concentration of monomeric actin to a limited extent [18]. Destrin, on the other hand, rapidly depolymerizes F-actin in a stoichiometric manner [22]. A putative nuclear transport signal was detected in cofilin and in destrin/ADF [19, 23]. Cofilin was shown to be transported, together with actin, in nuclei of fibroblastic and muscle cultured cells incubated in the presence of dimethyl sulfoxide (DMSO) or after heat shock treatment. In the nucleus they form actin/cofilin paracrystal-like structures called actin/cofilin rods [20, 24]. Recently, Ono *et al.* [25] demonstrated that destrin/ADF is also translocated into nuclear actin rod structures in DMSO-treated cells. Cofilin and destrin are phosphorylated under control conditions [26, 27, 28]. It has been shown that stresses such as 10% DMSO or heat shock provoke, in cultured fibroblastic cells, dephosphorylation of cofilin [28]. This has led to the hypothesis that dephosphorylation of cofilin might be the signal leading to its nuclear accumulation [28].

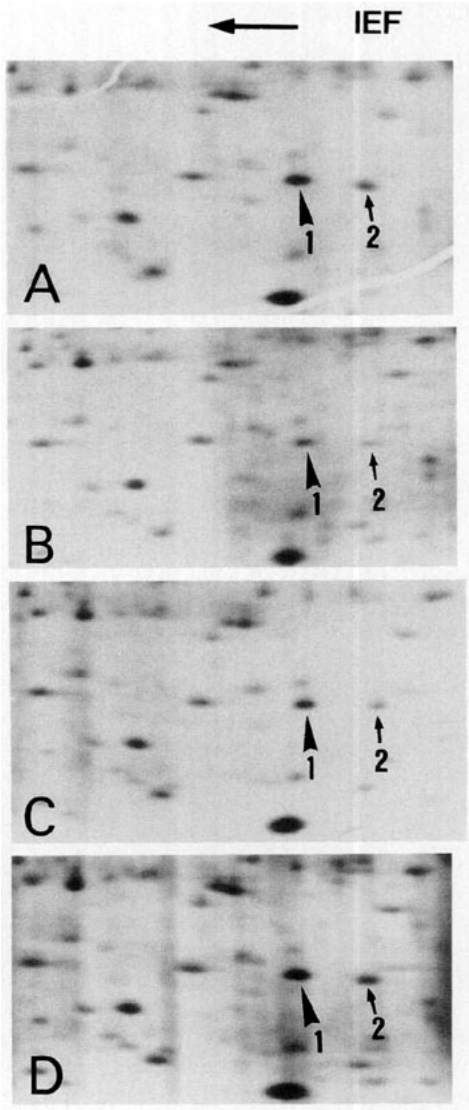


FIG. 1. Radioautographic detail of [^{35}S]methionine-labeled proteins extracted from thyroid cells and separated by two-dimensional electrophoresis (IEF/SDS–PAGE). A characteristic section of the 2-D gel was chosen to illustrate the effect of TSH, TPA, and EGF on the labeling of proteins p21 (spot 1) and p19 (spot 2). The acidic end of the gel is to the left. After 4 days of culture [^{35}S]methionine (600 μCi/ml) was added to each petri dish together with 1 mU/ml TSH (B), 10 ng/ml TPA (C), or 25 ng/ml EGF (D) and the culture was pursued for 1 h. (A) No agent added. Sample preparation and 2-D gel electrophoresis (IEF/SDS–PAGE) were performed as described under Materials and Methods. The same amount of total protein radioactivity was applied on each gel.

In the present work p21 and p19, two regulated proteins in dog thyroid cells, have been identified as being cofilin and destrin/ADF. Their regulation by TSH, the major element controlling thyroid function and growth

in vivo, has been characterized. The effect, on the properties of these proteins, of the two other thyroid mitogenic agents TPA and EGF and of the cellular stress agent DMSO has also been investigated.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, MCDB104 medium, penicillin, streptomycin, and amphotericin B (fungizone) were obtained from Flow Laboratories (Irvine, UK). Bovine insulin, TPA, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St Louis, MO). Bovine TSH was from Armour Pharmaceutical Co. (Chicago, IL). Forskolin was from Calbiochem (La Jolla, CA). Servalyte, pH 5–7, pH 3–10, pH 2–11, and leupeptin were obtained from Serva (Heidelberg, Germany), DEAE Sephacel and Ampholine, pH 5–7, were from Pharmacia LKB (Uppsala, Sweden). [^{35}S]methionine, [^{32}P]orthophosphate, and ^{125}I-protein A were from Amersham International (Buckinghamshire, UK). Bovine trypsin was purchased from Boehringer-Mannheim (Mannheim, Germany). Soluene 350 and Insta Fluor were from Packard Instrument Co. (Rockville, USA).

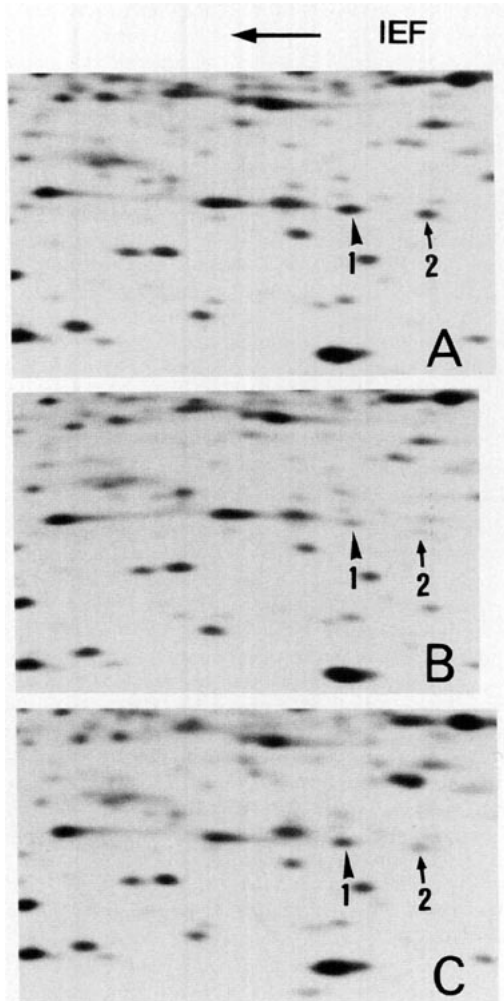


FIG. 2. Radioautographic detail of [^{35}S]methionine-labeled proteins extracted from thyroid tissue and separated by two-dimensional electrophoresis (IEF/SDS–PAGE). The acidic end of the gel is to the left. Thyroid tissue slices were incubated for 3 h with [^{35}S]methionine (150 μCi/ml) in the presence of 10 mU/ml TSH (B) or 5 μM TPA (C). (A) No agent added. Spot 1, p21; spot 2, p19. The same amount of total protein radioactivity was applied on each gel. The section of the 2-D gel presented in this figure is identical to the one shown in Fig. 1.

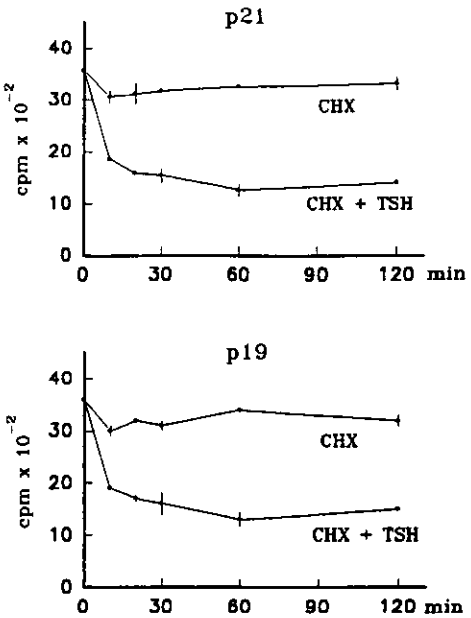


FIG. 3. Effect of cycloheximide on [^{35}S]methionine content of p21 and p19. Thyroid cells were labeled for 6 h with [^{35}S]methionine (330 μCi/ml) in order to label neo-synthesized proteins (0 min). They were then washed free of labeled methionine and incubated in the presence of cycloheximide (10 μg/ml) with or without TSH (1 mU/ml) for different periods of time. Samples of the cell lysates containing an equal number of counts per min (5×10^6 cpm) were subjected to 2-D gel electrophoresis (IEF/SDS–PAGE). Radiolabeled proteins were visualized by autoradiography, excised from the gels, and the amounts of radioactivity were measured as described under 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 two gels and quantitated. This value (37 ± 4 cpm) was subtracted from the counts per minute measured in [^{35}S]methionine-labeled p21 (spot 1) and p19 (spot 2). Values are expressed as mean ± range of duplicate assays.

Primary cultures. Dog thyrocytes were cultured in monolayer (2×10^4 cells/cm^2) in the following medium: DMEM + Ham's F12 medium + MCDB104 medium (2:1:1, v/v/v) supplemented by 5 μg/ml insulin, 40 μg/ml ascorbic acid, and antibiotics [9]. After 4 days of culture, the cells were incubated with [^{32}P]phosphate (1 mCi/ml) or with [^{35}S]-methionine (150 to 330 μCi/ml) for different periods of time. For ^{32}P radioactive labeling, the labeling medium was Eagle's minimal essential medium containing 9 μM KH_2PO_4, 15 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5 μg/ml insulin, and 2 mM glutamine.

Incubation of thyroid slices. Dog thyroid slices of about 0.2 mm were prepared as described previously [1] and were incubated (10 mg of wet tissue per flask) at 37°C, under 95% O_2 and 5% CO_2 in 1 ml Krebs–Ringer bicarbonate (KRB) buffer containing 8 mM glucose and 1 g/liter bovine serum albumin. The slices were incubated with [^{35}S]methionine (150 μCi/ml) for 3 h.

Sample preparation. After incubation, thyroid slices were homogenized in small glass grinders in lysis buffer (60 μl lysis buffer/1 mg wet

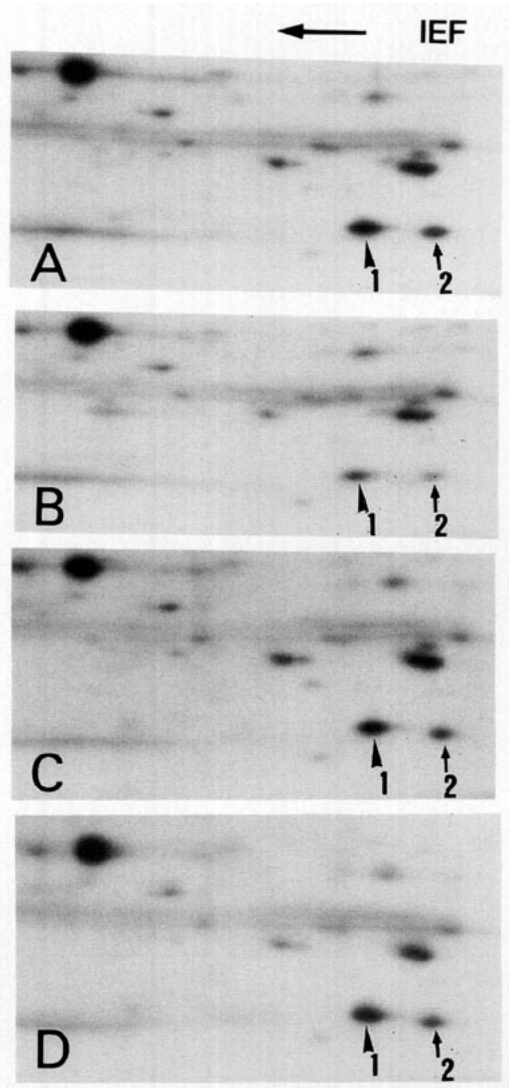


FIG. 4. Radioautographic detail of ^{32}P-labeled proteins extracted from thyroid cells and separated by two-dimensional electrophoresis (IEF/SDS–PAGE). A characteristic section of the two-dimensional pattern of proteins was chosen to illustrate the effect of TSH, TPA, or EGF on [^{32}P]phosphate incorporation into proteins p21 (spot 1) and p19 (spot 2). The acidic end of the gel is to the left. Thyroid cells were labeled for 8 h with $^{32}PO_4$ (1 mCi/ml). 1 mU/ml TSH (B), 10 ng/ml TPA (C), or 25 ng/ml EGF (D) was added 20 min before the end of the culture. (A) No agent added.

weight tissue) containing 9.5 *M* urea, 2% w/v Nonidet-P40, 2% carrier ampholytes, pH 7–9, and 100 m*M* dithiothreitol (DTT) [29]. For cell cultures 240 μl of the same lysis buffer was added to each petri dish (3.5 cm diameter). The cells were then scraped with a plastic rod. Following homogenization the samples were centrifuged for 2 min in a microfuge to remove DNA and insoluble cell debris. Total protein radioactivity was determined as described [1].

2-D gel electrophoresis. In the first dimension proteins were separated on cylindrical gels for (i) IEF with 1.6% Servalytes, pH 5–7, 1.6% Ampholines, pH 5–7, and 0.8% Servalytes, pH 2–11, as described previously [1]; (ii) nonequilibrium pH gradient electrophoresis (NEPHGE) with 2% Servalytes, pH 3–10, as described [29]. In the second dimension, proteins were separated according to molecular mass on sodium dodecyl sulfate, linear gradient (6–16%) polyacrylamide slab gels (total length of the separation gel = 18 cm) as described previously [1]. Gels containing ^{32}P-labeled proteins were submitted to autoradiographic exposure using Amersham β max films, whereas fluorography was performed on gels containing ^{35}S-labeled proteins using Amersham MP films. Silver staining of the gels was performed as described in [30].

Quantitative estimation of [^{32}P]phosphate or of [^{35}S]methionine labeling of proteins. The area of the two-dimensional gel corresponding to the spot of interest seen on the autoradiograph or on the fluorograph was excised and swollen in 200 μl of H_2O overnight. The protein was then solubilized in 1 ml of Soluene 350 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.

Microsequencing of proteins. Dog thyroid tissue (5 g) was homogenized in 8 ml of 20 m*M* Tris–HCl, pH 8.3, 0.1 m*M* EDTA, 8.7% glycerol, 21 m*M* β-mercaptoethanol, 0.5 μg/ml leupeptin, 5 μg/ml PMSF with a glass-glass homogenizer at 0°C and centrifuged for 20 min at 4°C and 30,000*g* with a Sorvall SS34 rotor and then for 1 h at 4°C and 100,000*g* with a Beckman 75 Ti rotor. The supernatant was applied to a DEAE-Sephacel column (2.6 × 7.5 cm) and the flow-through solution was collected. Proteins were then eluted with different NaCl concentrations in the same buffer as the one used for tissue homogenization (pH 8.3). One of the proteins of interest was found in the flow-through solution (p19) and the other (p21) in the 0.05 *M* NaCl eluting solution. Each solution was then concentrated with Amicon 202 and PM10 membrane as described by the manufacturer (Amicon Co., Danvers, MA) at 4°C. The proteins were then precipitated with TCA (6% final) in the presence of 125 μg/ml sodium deoxycholate [31]. The precipitated proteins were dissolved in lysis buffer and separated on IEF-2D gel electrophoresis. After detection of proteins with Coomassie brillant blue staining, proteins of interest were excised from the gels (about 25 gels) and digested with bovine trypsin as described [32]. The resulting peptides were separated by reverse-phase chromatography on a C-18 column (8P 300RP, Chrompack, Netherlands). Several peptides were sequenced using a 477A pulsed liquid-phase sequenator (Applied Biosystems, USA).

Blotting and immunodetection of proteins. After separation by 2D-gel electrophoresis, proteins were transferred to a nitrocellulose membrane, PH79 (Schleicher & Schüell, Dassel, Germany) for 16 h at 60 V and 4°C [1]. Monospecific rabbit polyclonal antibody against porcine cofilin [24] was used at a 1:50 dilution, whereas rabbit polyclonal antibody against chicken actin depolymerizing factor (ADF) provided by Dr. Bamburg (Colorado State University, Fort Collins, CO) was used at a 1:100 dilution. ^{125}I-protein A (5×10^6 dpm/ml) was used as a secondary reagent for development by autoradiography. Autoradiography before immunoreaction was done by direct contact of the nitrocellulose sheet with an Amersham MP film, which allows detection of ^{35}S-labeled proteins. After immunoreaction, an exposed and processed film was placed between the nitrocellulose sheet and the film in order to prevent the formation of spots by ^{35}S-labeled proteins but to allow the recording of spots due to ^{125}I-protein A complexes. An intensifying screen (Siemens Special) further increases the intensity of the spots due to ^{125}I.

Indirect immunofluorescence. For cofilin staining, cells in petri dishes (2×10^4 cells/cm^2) were fixed with methanol for 10 min at −20°C, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, and blocked with 5% sheep serum for 30 min. Cells were then incubated overnight at 4°C with polyclonal antibody against cofilin at 1:10 dilution in PBS containing 0.1% bovine serum albumin (PBS–BSA), washed with PBS–BSA and incubated for 2 h at room temperature with fluorescein-conjugated donkey anti-rabbit immunoglobulins (Amersham) at 1:50 dilution in PBS–BSA. For actin staining, cells were either fixed with paraformaldehyde (90 sec at 4°C) and then methanol (10 min at −20°C) or extracted with deter-

TABLE 1

Partial Amino Acid Sequences Generated from Dog Thyroid p19 and p21 Isolated from Two-Dimensional Gels

Protein	Sequences in one-letter notation	Residues in identified protein	Protein name	References
p19	DASFETK	86–92	Destrin/ADF	[19, 20, 42]
	HE(X)QANGPEDL	133–143	(porcine and chicken)	
	EG(X)PV	161–165		
p21	YAYL	82–85	Cofilin	[23, 43]
	IFWAPE(X)APL	102–111	(porcine and murine)	

Note. The proteins p19 and p21 were isolated from 2-D gels (IEF/SDS–PAGE) of, respectively, the flow-through fraction and the 0.05 *M* NaCl eluting fraction of DEAE-Sephacel chromatography of total thyroid extracts. (X) in the sequences indicates the positions where residues could not be identified.

gent (0.1% Triton X-100 in 10 m*M* phosphate buffer, pH 7.2, with 5 m*M* $MgCl_2$ and 10 m*M* NaCl) for 10 min at 4°C before fixation with methanol (10 min at −20°C). Monoclonal anti-actin (Boehringer-Mannheim) was used at 10 μg/ml overnight at 4°C and revealed using biotinylated sheep anti-mouse immunoglobulin (Amersham) and fluorescein streptavidin (Amersham). Cells were viewed with Leitz or Zeiss epifluorescence microscopes (50X oil immersion lens). Microphotographs were taken using 400 ISO Fujichrome films with fixed exposures.

RESULTS

Effect of a Short Exposure Time of Thyroid Cells to TSH, TPA, and EGF on [^{35}S]Methionine Incorporation into p21 and p19

After 4 days of culture in defined medium, in the absence of serum, dog thyroid cells are quiescent and form a well-spread monolayer. At this time, they were exposed for 1 h to TSH, TPA, or EGF in the presence of [^{35}S]methionine. The proteins were then resolved by 2-D gel electrophoresis (IEF/SDS–PAGE). Figure 1B shows that TSH strongly decreased [^{35}S]methionine incorporation into p21 (spot 1) and p19 (spot 2). This effect of TSH was reproduced by 10^{-5} *M* forskolin, a general activator of adenylate cyclase [33], indicating that it was mediated through an elevation in intracellular cyclic AMP concentration (not shown). At this time point, TPA had a weaker effect on [^{35}S]methionine incorporation into these proteins (Fig. 1C), whereas EGF did not seem to modify their labeling at all (Fig. 1D). The apparent p*I* values of p21 (spot 1) and p19 (spot 2) determined in the IEF/SDS–PAGE system were 6.7 and 6.85, respectively.

p21 and p19 Are Present and Are Regulated in Dog Thyroid Tissue

Figure 2A shows that dog thyroid tissue slices contain two proteins, p21 (spot 1) and p19 (spot 2), which present the same characteristic migration in 2-D gels as p21 and p19 extracted from primary culture of dog thyroid cells. Identity between these two sets of proteins was further demonstrated by simultaneous separation, in the same 2-D gel, of [^{35}S]methionine-labeled proteins extracted from cultured cells and of [^{35}S]methionine-labeled proteins extracted from tissue slices: p21 and p19 [^{35}S]methionine-labeled proteins from both sources were indistinguishable; they comigrate at exactly the same place in the 2-D gel (not shown).

Figure 2B shows that, in tissue slices as in cultured cells, TSH after 3 h of action strongly decreased [^{35}S]-methionine incorporation into p21 and p19. This effect of TSH was reproduced by 10^{-5} *M* forskolin (not shown). In this system also, TPA decreased to a lesser extent than TSH [^{35}S]methionine incorporation into these proteins (Fig. 2C), whereas EGF (250 ng/ml) had no effect (not shown). These results demonstrate that p21 and p19 are present in the thyroid gland and are not neo-synthesized as a result of the cell culture process. They also suggest that regulation of these proteins, at least by TSH, has physiological significance. Another consequence of these observations is that thyroid glands instead of primary cultures of thyroid cells can be used as the starting material for the purification of these proteins.

Effect of Cycloheximide on [^{35}S]Methionine Content of p21 and p19

Silver staining of 2-D gels (IEF/SDS–PAGE) after separation of protein extracts from cells treated with TSH revealed that this agent not only strongly decreased the [^{35}S]methionine incorporation into these proteins but that it also decreased their chemical amount (not shown). As this might be due to an effect of TSH on the half-life of these proteins, the [^{35}S]-methionine content of p21 and p19 was measured after exposure of the prelabeled cells, for different periods of time, to cycloheximide and TSH. Labeling of total proteins did not decrease even after 120 min of incubation

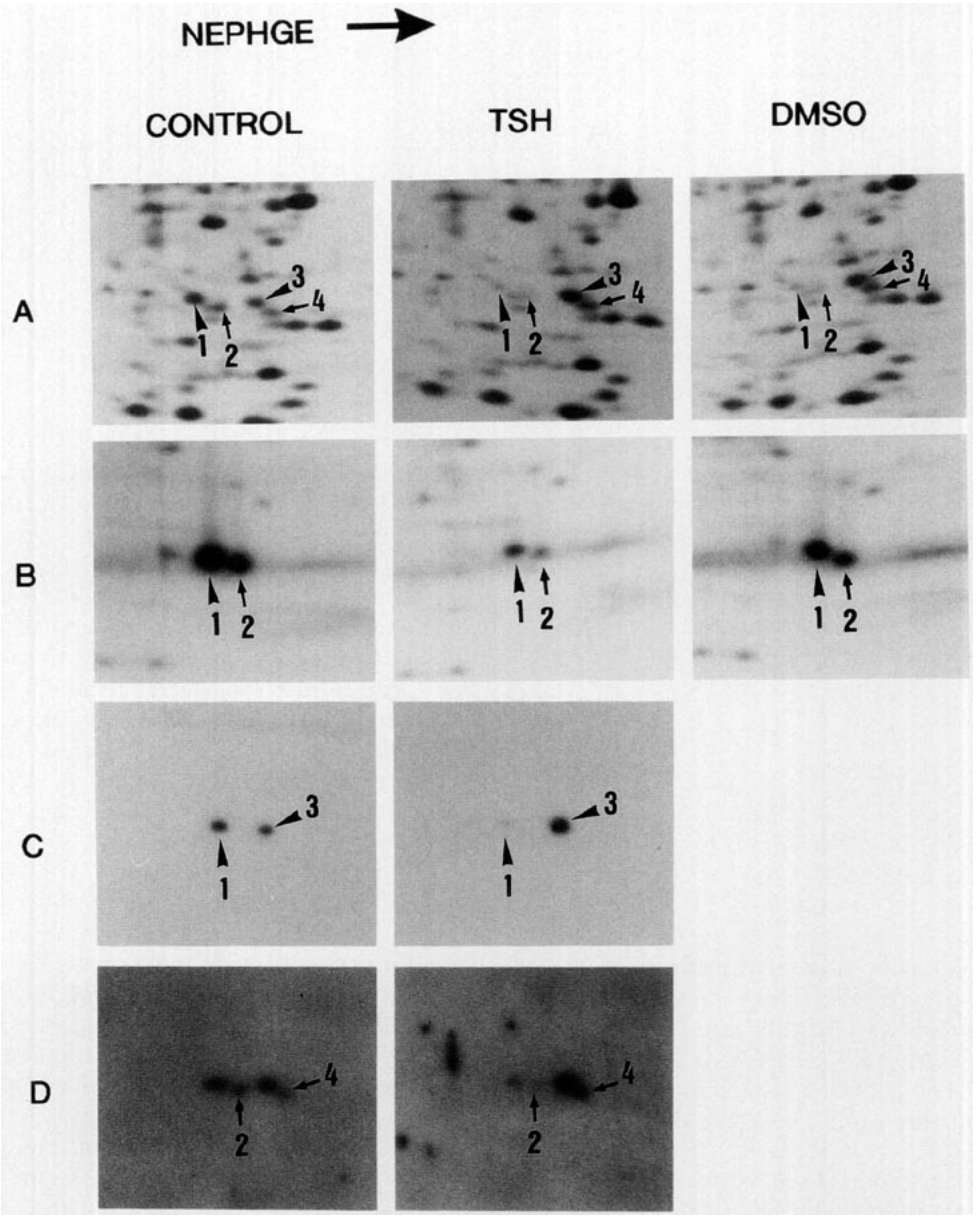


FIG. 5. Two-dimensional gel electrophoresis (NEPHGE/SDS–PAGE) analysis of cofilin (p21) and destrin (p19). Cells labeled with [^{35}S]methionine (150 μCi/ml) for 24 h (lane A) or with [^{32}P]phosphate (1 mCi/ml) for 3 h (lane B) and incubated for the last hour of the culture period with TSH (1 mU/ml) or DMSO (10%) were lysed and subjected to 2-D gel electrophoresis (NEPHGE/SDS–PAGE) and fluorography (lane A) or autoradiography (lane B). Only a relevant section of the 2-D gel is shown. The acidic end of the gel is to the left. Lanes C and D, autoradiographs of the same relevant section of the 2-D gel after transfer of [^{35}S]methionine-labeled proteins to nitrocellulose membranes and specific immunoreaction, first against cofilin (lane C) and then against destrin/ADF (lane D). ^{125}I radiation was recorded by using a screen that stops ^{35}S radiation as described under Materials and Methods. Spots 1 and 3, cofilin; spots 2 and 4, destrin.

of the prelabeled cells in the absence of [^{35}S]methionine and the presence of cycloheximide, with or without TSH (not shown). Figure 3 shows that the presence of cycloheximide alone (10 to 120 min) did not affect [^{35}S]-methionine content of p21 and p19, indicating that these proteins are not labile. Figure 3 also shows that the presence of TSH in addition to cycloheximide provoked a very rapid decrease in the labeling of both proteins. After 20 min of exposure of the cells to TSH and cycloheximide only 40% of the initial labeling remained in both of them. Longer exposure times of the cells to these agents did not affect the amount of radioactivity present in p21 and p19 any further (Fig. 3), suggesting that TSH was acting on another parameter than the half-life of these proteins. 2-D gel electrophoresis of total proteins secreted in the cell culture medium did not

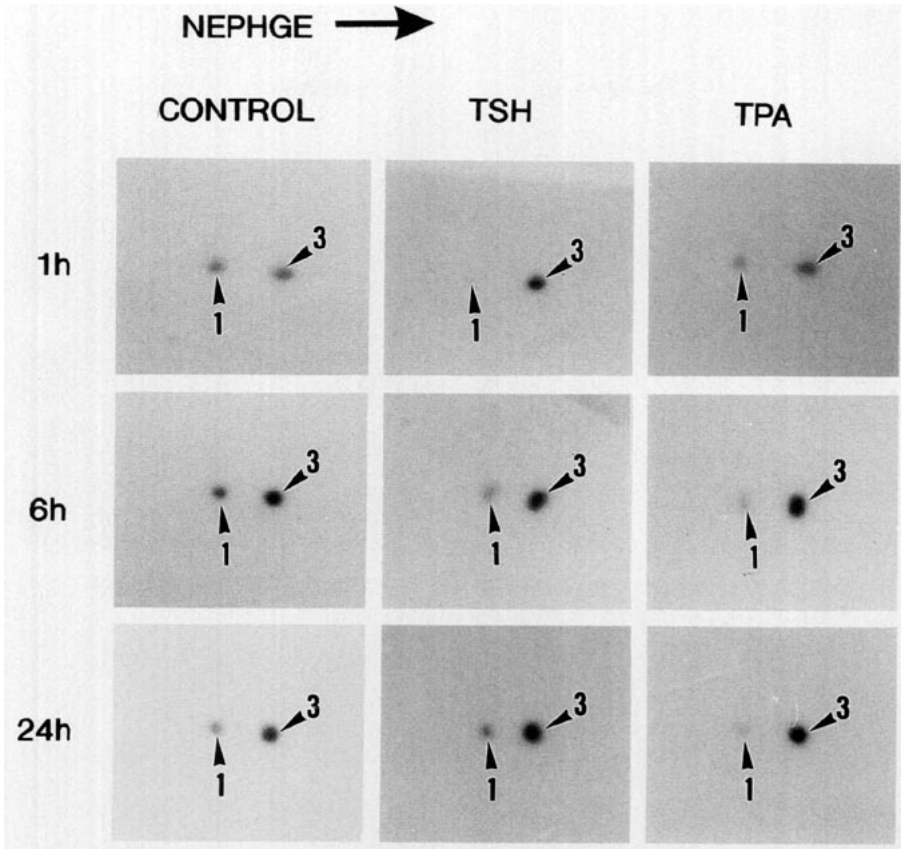


FIG. 6. Kinetics of cofilin dephosphorylation in response to different agents. Anti-cofilin immunoblots after 2-D gel electrophoresis (NEPHGE/SDS-PAGE) of whole cell extracts from dog thyrocytes stimulated for the times indicated on the figure with TSH (1 mU/ml), TPA (10 ng/ml), or EGF (25 ng/ml). The same amount of protein was loaded on each gel. The acidic end of the gel is to the left.

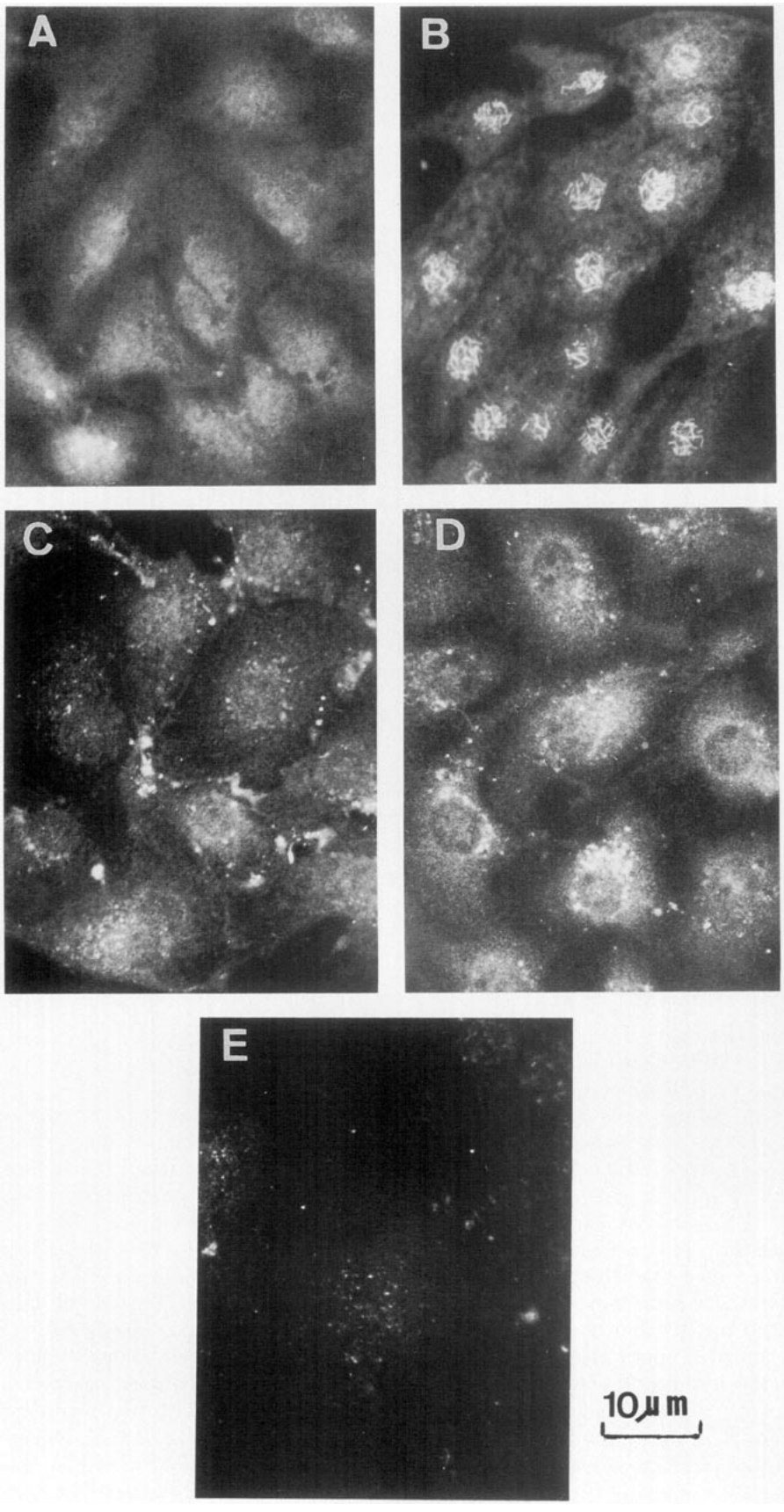
reveal TSH-induced secretion of p21 and p19 (not shown).

p21 and p19 Are Phosphorylated Proteins

2-D gel separation (IEF/SDS-PAGE) of total proteins extracted from thyroid cells which had been incubated in the presence of [^{32}P]phosphate followed by silver staining and autoradiography revealed that p21 (spot 1) and p19 (spot 2) were phosphorylated proteins (Fig. 4A). A 20-min exposure of the cells to TSH (Fig. 4B) but not to TPA (Fig. 4C) or to EGF (Fig. 4D) strongly decreased [^{32}P]phosphate incorporation into p21 and p19. The fact that, at this time point, TPA effect was not detectable suggests a slower kinetics of action. The effect of TSH was reproduced by 10^{-5} *M* forskolin (not shown) and was further documented by directly counting p21 and p19 spots after excision from 2-D gels: a decrease of approximately 60% in the labeling of both proteins was observed after TSH or forskolin action. The demonstration that p21 and p19 are phosphorylated proteins raised the possibility that the decrease in the [^{35}S]methionine or [^{32}P]phosphate labeling of these proteins in response to TSH might be due to their dephosphorylation accompanied by an increase in isoelectric pH and a shift outside the pH gradient of the gel (pH range 5–7).

Identification of p21 and p19 after Their Microsequencing

Table 1 shows the sequence of several peptides generated during digestion with trypsin of purified p21 and p19 and then separated by reverse-phase high performance liquid chromatography (HPLC). The three sequences generated from p19 (Table 1) matched sequences present in 19-kDa porcine destrin and in the 19-kDa chicken ADF stored in the Swiss-Prot protein sequence database. Porcine destrin and chick ADF are over 95% identical based upon sequence analysis of cloned cDNAs, have identical actin depolymerizing activities and are believed to be the same protein (reviewed in [21]). The two peptides generated from p21 (Table 1) are identical to sequences of porcine or murine cofilin stored in the Swiss-Prot protein sequence database. The identity of thyroid p21 and p19 has been fur-


A
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ther verified after 2-D gel separation (IEF/SDS–PAGE) by cross-reactivity with specific antibodies against brain cofilin and chicken ADF (not shown).

Thyroid Cofilin and Destrin Are Dephosphorylated in Response to TSH and DMSO

In several types of cells, cofilin and destrin have been shown, using 2-D gel electrophoresis with a nonequilibrium pH gradient electrophoresis in the first dimension (NEPHGE/SDS–PAGE), to exist in two forms: a phosphorylated and an unphosphorylated one [26, 27, 28]. In both cases, dephosphorylation resulted in a pH shift toward more basic values. This led us to investigate the characteristics of thyroid cofilin (p21) and destrin (p19) using the same 2-D gel electrophoresis procedure.

Dog thyroid cells were incubated in the presence of [^{35}S]methionine or [^{32}P]phosphate with or without TSH and total protein extracts were separated by 2-D gel electrophoresis (NEPHGE/SDS–PAGE). Migration characteristics of cofilin (p21) and destrin (p19) in this experimental system were revealed by cross-reactivity with specific antibodies against brain cofilin (Fig. 5, lane C) and chicken ADF (Fig. 5, lane D). Under control conditions [^{35}S]-labeled cofilin and destrin appear as two spots: spots 1, 3 and 2, 4, respectively (Fig. 5, lane A). In contrast [^{32}P]phosphate-labeled cofilin and destrin both appear as a single spot: spots 1 and 2, respectively (Fig. 5, lane B). Thus spots 1 and 2 represent the phosphorylated form of cofilin and destrin, whereas spots 3 and 4 represent their dephosphorylated form. Exposure of the cells, for 1 h, to TSH provoked a strong decrease in the intensity of [^{35}S]methionine-labeled spots 1 and 2 with a concomitant increase in the intensity of spots 3 and 4 (Fig. 5, lane A) and a strong decrease in the intensity of [^{32}P]-labeled cofilin and destrin (respectively, spots 1 and 2 in Fig. 5, lane B). These results indicate that TSH provoked an almost complete dephosphorylation of both proteins. This effect of TSH was reproduced by 10^{-5} *M* forskolin (not shown). As, in cultured fibroblastic cells, Ohta *et al.* [28] demonstrated that stresses such as 10% DMSO provoked the dephosphorylation of cofilin, we studied the effect of this agent on cofilin and destrin phosphorylation in dog thyroid cells. Figure 5 (lanes A and B) shows that exposure of thyroid cells for 1 h to DMSO provoked, as with TSH, a marked dephosphorylation of cofilin and destrin.

Kinetics of Cofilin and Destrin Dephosphorylation in Response to TSH, TPA, and EGF

Whole cell extracts from dog thyrocytes were separated by 2-D gel electrophoresis (NEPHGE/SDS–PAGE) after stimulation by TSH, TPA, or EGF and both proteins were revealed by cross-reactivity with anti-cofilin and anti-ADF antibodies. Figure 6 shows that TSH induced a faster dephosphorylation of cofilin than TPA. EGF was without effect on this parameter (not shown). After 1 h of action of TSH, the dephosphorylation of cofilin was almost complete, whereas TPA had only a weak effect. After 6 h of action, both agents affected the dephosphorylation of cofilin to the same extent. After 24 h of action, TSH did not significantly modify cofilin phosphorylation, whereas TPA induced its almost complete dephosphorylation. The same results were observed for the effect of TSH and TPA on destrin dephosphorylation (not shown). EGF had no effect on destrin dephosphorylation at 1 and 6 h but provoked some dephosphorylation of this protein after 24 h of action (not shown).

These data are in accordance with the results obtained using 2-D gel electrophoresis with isoelectric focusing electrophoresis in the first dimension (IEF/SDS–PAGE) which are presented in Figs. 1 to 4. In this 2-D gel system only the more acidic form, i.e., the phosphorylated form, of cofilin and destrin is present. The decrease in [^{35}S]methionine- or [^{32}P]phosphate-labeling of cofilin and destrin observed after IEF/SDS–PAGE separation of proteins is thus due to dephosphorylation rather than to a decrease in the biosynthesis of these proteins.

DMSO but Not TSH, TPA, or EGF Induces the Formation of Intranuclear Cofilin Rods

It has been shown that exposure of fibroblastic cells to cellular stress agents such as 10% DMSO induces not only dephosphorylation of cofilin but also the formation of intranuclear actin/cofilin paracrystal-like structures or rods which were strongly stained by porcine anti-cofilin antibody [24, 28]. Using the same porcine anti-cofilin antibody we have shown that, whereas 10% DMSO induced, in dog thyrocytes as in fibroblasts, the formation of intranuclear actin/cofilin rods (Fig. 7B), this was not observed in response to TSH (Fig. 7C), TPA (Fig. 7D), or EGF (not shown), although the action of these agents was studied at several time points (20 min, 1, 3, 6, and 24 h). In control thyrocytes (Fig. 7A) cofilin had a diffuse distribution, as the one observed in fibroblasts [24]. In the presence of 10% DMSO, bright staining appeared in the nuclei, mostly forming rod structures. This suggests that, as in DMSO-treated fibroblasts [25], cofilin moved into the nuclei. TSH induced a stronger staining along some areas of the cell periphery (Fig. 7C), whereas TPA provoked a preferential perinuclear

FIG. 7. Indirect immunofluorescent localization of cofilin. Dog thyrocytes were fixed with methanol and incubated with antibody against cofilin (A–D) or with this antibody preincubated with partially purified cofilin (E) as described under Materials and Methods. Before fixation, cells were cultured for 1 h in control medium (A and E) or with 10% DMSO (B), 1 mU/ml TSH (C), or 10 ng/ml TPA (D).

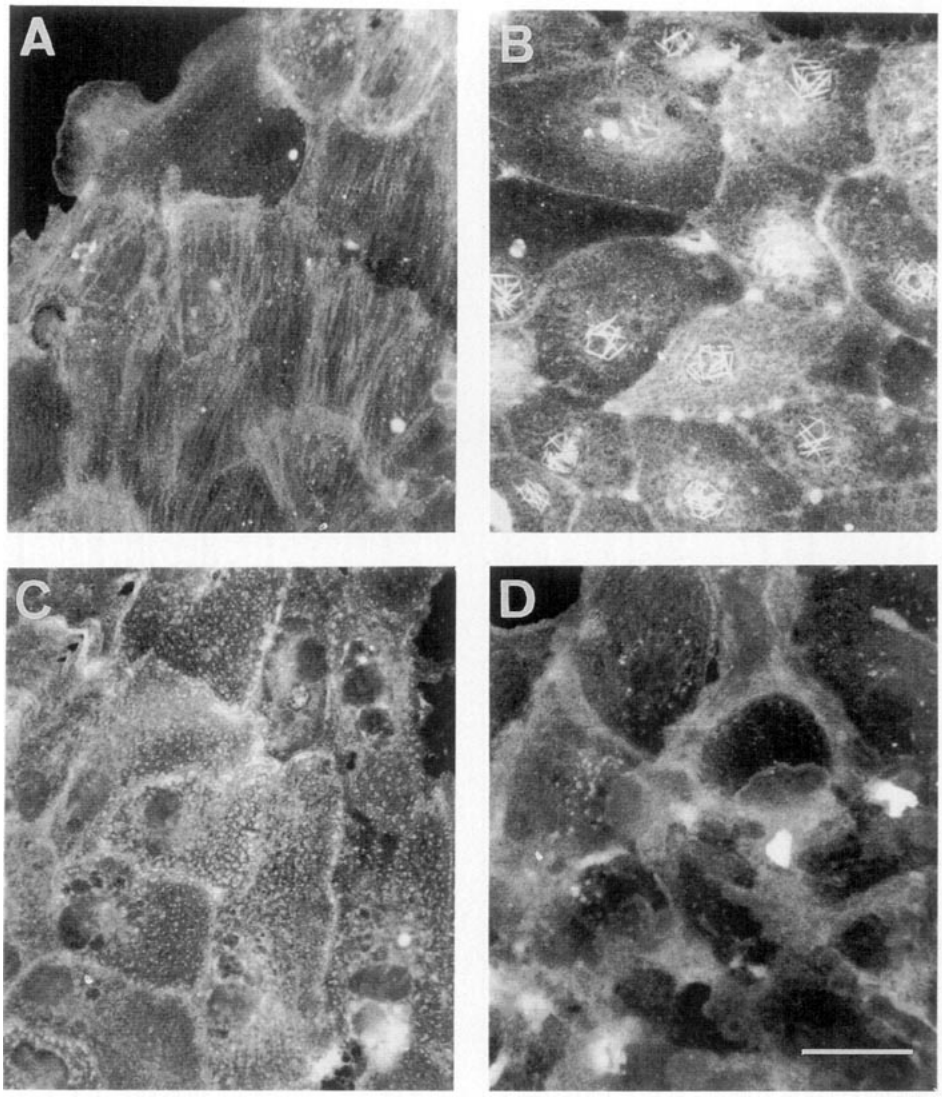

FIG. 8. Indirect immunofluorescent localization of actin. Dog thyrocytes were fixed with paraformaldehyde and then with methanol and incubated with an antibody against actin as described under Materials and Methods. Before fixation, the cells were cultured in control medium (A) or for 1 h with 10% DMSO (B), 1 mU/ml TSH (C), or 10 ng/ml TPA (D). Bar, 10 μm.

staining (Fig. 7D). Thus, although TSH and TPA as DMSO induced dephosphorylation of cofilin, only DMSO provoked the formation of nuclear cofilin rods. Preabsorption of antibody against cofilin with partially purified cofilin blocked almost completely the staining of control cells (Fig. 7E).

TSH, TPA, and DMSO Distinctly Modify the Distribution of Actin Microfilaments (Figs. 8 and 9)

Indirect immunofluorescent labeling of actin in control cells showed an abundant stress fiber network throughout the entire cell length, which was superimposed to a more diffuse staining (Fig. 8A). Stress fibers were better visualized in control cells extracted with a Triton X-100 hypotonic buffer before fixation (Fig. 9A). Within 1 h, DMSO (Fig. 8B) mimicked the previously shown [5, 34] TSH effect (Fig. 8C), i.e., the disruption of the stress fiber network, and the redistribution of actin at the cell periphery and in finely punctate dots. However, as also shown by cofilin immunostaining (Fig. 7), DMSO (Fig. 8B), but not TSH (Fig. 8C), induced the appearance of intranuclear rod structures, confirming that they contain both actin and cofilin [24]. Extraction of the cells with the Triton X-100 hypotonic buffer revealed that DMSO (Fig. 9B), like TSH (Fig. 9C), also induced the reorganization of actin in a fine "microtrabecular" network throughout the cytoplasm. A similar detergent-resistant network has recently been evidenced after TSH treatment by Deery and Heath using

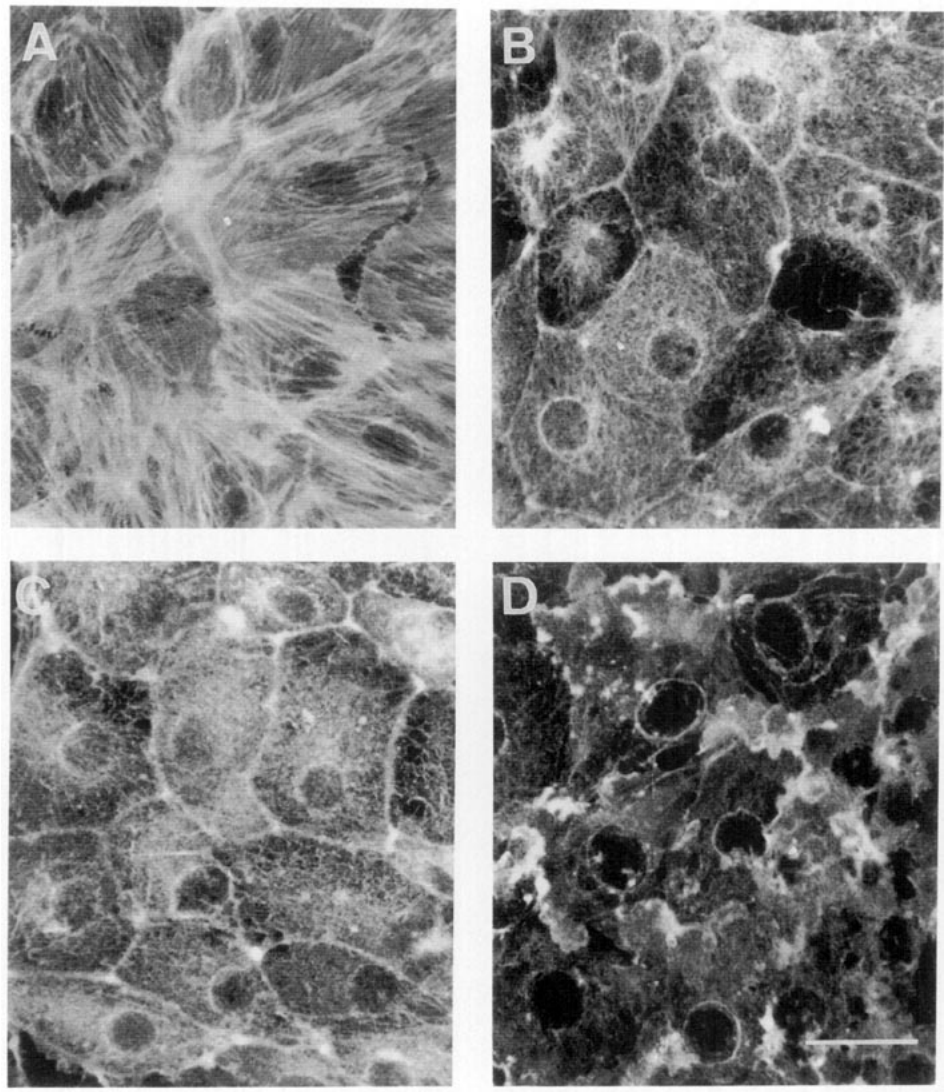

FIG. 9. Immunofluorescent detection of actin in detergent-extracted cytoskeleton. Dog thyrocytes were extracted with a Triton X-100 hypotonic buffer before fixation with methanol and immunofluorescent detection of actin as described under Materials and Methods. Cells were cultured in control medium (A) or for 1 h with 10% DMSO (B), 1 mU/ml TSH (C), or 10 ng/ml TPA (D). Bar, 10 μm.

a myosin antibody [6]. Unlike the stress fiber and microtrabecular networks, the actin/cofilin nuclear rods were extracted by the Triton X-100 hypotonic buffer (Fig. 9B). As previously shown [5, 6], TPA also induced the disorganization of actin stress fibers (Figs. 8D and 9D), although more actin bundles persisted after TPA treatment than in response to TSH (not shown). Actin redistributed and decorated very prominent lamellar membrane structures illustrating dramatic blebbing and ruffling of membrane regions mostly at the cell periphery (Figs. 8D and 9D). Interestingly, while cofilin occasionally associated with some membrane ruffles in the case of TSH stimulation (Fig. 7C), the prominent TPA-induced membrane protrusions were never stained with cofilin (Fig. 7D). This indicates that cofilin could participate to actin redistribution at the cell periphery in the case of TSH but not of TPA treatment. The disorganization of stress fibers induced by TSH and TPA preceded quite different morphological changes. Within a few hours, TSH progressively promoted cytoplasmic retraction and cell arborization, whereas TPA-treated cells adopted a distorted, irregular outline with many lamellar protrusions at their periphery, followed by disruption of confluence and increased motility [5, 8].

DISCUSSION

In a previous work we have shown, using separation of total cellular proteins by 2-D gel electrophoresis (IEF/SDS–PAGE), that dog thyroid cells in culture contain two proteins, p21 and p19, which are regulated

by TSH, TPA, and EGF [12, 13, 17]. In this work, these proteins have been purified by the same electrophoretic technique. Their partial sequencing has revealed that p21 is identical to cofilin, whereas p19 is identical to destrin/ADF, two closely related and widely distributed actin-binding proteins which are able to depolymerize actin filaments *in vitro* [35, 36]. This identification of p21 and p19 has been further verified by cross-reactivity with specific antibodies against brain cofilin and chicken ADF.

The use of a different 2-D gel electrophoretic technique, i.e., with a nonequilibrium pH gradient electrophoresis in the first dimension (NEPHGE/SDS–PAGE) led us to demonstrate that, in cultured thyroid cells as in cultured fibroblasts [28], myocytes, and PC12 cells [26, 27, 37], cofilin and destrin/ADF are present, under control conditions, in two forms: a phosphorylated and an unphosphorylated one. TSH, through cyclic AMP, provoked a very rapid dephosphorylation of these two proteins. This was demonstrated by a decrease in [^{35}S]methionine incorporation and in [^{32}P]-phosphate incorporation into the phosphorylated form and by an increase in [^{35}S]methionine incorporation into the unphosphorylated forms of both proteins. This effect of TSH was already noticed after 10 min and was maximal after 20 min of action of this hormone. TPA also provoked the dephosphorylation of cofilin and destrin but with a much slower kinetics: its effect was weakly noticeable after 1 h of action and was of the same magnitude as that induced by TSH after 6 h of action. EGF affected only the phosphorylation of destrin/ADF and this at a very late time point (24 h). It is of significance that only the dephosphorylated form of destrin/ADF has been reported to be able to affect the extent of actin assembly [26, 27]. It is likely that this is also true for cofilin and that only its dephosphorylated form is able to interact with actin [27].

As shown in this and previous studies [5, 6], in the dog thyroid cell, TPA acting through protein kinase C and TSH via cyclic AMP trigger profound but distinct morphological changes that are preceded by a rapid disruption of actin-containing stress fibers. This was already observed 20 min after the addition of both agents [5]. The dephosphorylation by TSH via cyclic AMP of destrin/ADF and of cofilin may thus play a role in the disruption of actin-containing stress fibers and in the reorganization of actin microfilaments induced by this hormone. In contrast, the delayed but more durable dephosphorylation of both actin-binding proteins in response to TPA is less likely involved in the rapid microfilament changes induced by this agent. It is worth noting that, like destrin in several cell lines [36], cofilin associates with some membrane ruffles in TSH-treated thyrocytes but not with the prominent actin-decorated membrane protrusions induced by TPA. This observation is also compatible with an involvement of cofilin at these sites of rapid actin reorganization when they are acutely induced by TSH but not when they are induced by TPA. Dephosphorylation of destrin/ADF in response to dibutyryl cAMP has been observed in astrocytes with a concomitant disruption of the cortical actin network [38]. This was accompanied by a dephosphorylation of the regulatory light chain of myosin. It has been suggested that the dephosphorylation of either or both proteins could lead to the disruption of microfilaments [5, 38, 39]. In the thyroid, TPA through kinase C as well as TSH through cyclic AMP also decrease the phosphate content of the myosin light chains [10, 40]. This was already observed after 15 min of action of these agents. It is thus possible that the dephosphorylation of myosin light chains plays a major role in the disruption of actin stress fibers induced by TPA in these cells. It is worth noting that EGF, which does not induce rapid dephosphorylation either of cofilin and destrin/ADF or of myosin light chains [10] in the thyroid cell, does not provoke acute morphological changes in these cells [5]. The delayed dephosphorylation of destrin/ADF induced by TPA and EGF and, to a lesser extent, by TSH could be linked to the long-term morphological changes induced by these agents, those induced by EGF and TPA being similar [8].

In cultured fibroblasts, cofilin has been shown to be a major component of intranuclear and cytoplasmic actin paracrystal-like structures or rods, which are formed when these cells are exposed to a variety of extracellular stimuli: heat shock or 10% dimethyl sulfoxide treatment induces intranuclear actin/cofilin rods, whereas incubation in salt buffers induces cytoplasmic rods [28]. In our work we show that, in thyroid cells, 10% DMSO treatment also induced the appearance of these intranuclear actin/cofilin rods but that these structures were not observed in response to TSH, forkolin, TPA, or EGF. In CHO cells but not in other cell lines forkolin and dibutyryl cAMP induce actin paracrystal-like structures close to the cell periphery [41]. As in thyroid cells, TSH like DMSO and with a slower kinetics TPA provoked a strong dephosphorylation of cofilin, our data do not support the hypothesis [28] that the dephosphorylation of cofilin might be correlated with its nuclear transport. Rather, the rapid dephosphorylation of cofilin and destrin induced by DMSO could explain the fact that this agent mimics the effect of TSH on actin reorganization besides its specific induction of nuclear actin/cofilin rods. It is worth noting that in cultured myotubes, 10% DMSO treatment induced the formation of nuclear actin/cofilin rods without affecting the proportion of the phosphorylated and unphosphorylated forms, also suggesting that dephosphorylation of cofilin is not implicated in its nuclear localization [37].

The significance for thyroid function of rapid effects of TSH and protein kinase C activators on microfilament organization remain to be established. In dog thy-

roid tissue, in response to TSH, but not to TPA, follicule cells form pseudopods and endocytose follicular colloid. This process mediated by contractile proteins is the first step in stimulated thyroid hormone secretion and could represent the *in vivo* equivalent of the phagocytic activity induced by TSH, but not by TPA, in cultured dog thyroid cells [6]. While stimulation of either A or C kinase leads to myosin light chain dephosphorylation and actin stress fiber disruption, it has been suggested that another event mediated by kinase A should be necessary for phagocytic activity [6]. We propose the dephosphorylation of cofilin and destrin acutely induced by TSH through cyclic AMP to be such events. The phosphatase(s) involved in these cyclic AMP-induced dephosphorylations remains to be defined.

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