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Forskolin and Camptothecin Induce a 30 kDa Protein Associated with Melatonin Production in Y79 Human Retinoblastoma Cells

Jina L. Janavs,[a] Jose C. Florez, Mary E. Pierce,[b] and Joseph S. Takahashi

NSF Center for Biological Timing, Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208-3520

The synthesis of melatonin in *Xenopus* retinas, chick and quail retinal cell cultures, and Y79 human retinoblastoma cells is stimulated by cAMP through a protein synthesis–dependent mechanism. In Y79 retinoblastoma cells, combined treatment with the RNA synthesis inhibitor camptothecin and agents that elevate cAMP, such as forskolin, causes a synergistic elevation of melatonin. Using two-dimensional gel analysis we have identified a 30 kDa cytosolic protein (p30) whose radiolabeling was consistently increased in parallel with increases in arylalkylamine *N*-acetyltransferase activity and melatonin production that were induced by forskolin and/or camptothecin. Pulse–chase experiments suggest that the elevation in radiolabeling of p30 is due to increased synthesis. Three candidate proteins found in the mammalian pineal, protein 14-3-3, malate dehydrogenase, and recoverin, do not comigrate with p30.

[Key words: melatonin, RNA synthesis, protein synthesis, two-dimensional gel electrophoresis, forskolin, cAMP, retinoblastoma, camptothecin, N-acetyltransferase]

Circadian melatonin synthesis is involved in regulating the rhythmic physiology of the retina in vertebrates (reviewed in Cahill et al., 1991). As in the pineal gland, retinal melatonin is synthesized from tryptophan by the sequential action of tryptophan hydroxylase (EC 1.14.16.4), arylalkylamine *N*-acetyltransferase (NAT; EC 2.3.1.5), and hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.87) (reviewed in Axelrod, 1974; Klein et al., 1981). Melatonin and the enzymes for its synthesis have been found in the retinas of a number of vertebrates (reviewed in Wiechmann, 1986; Besharse et al., 1988; Iuvone, 1990; Cahill et al., 1991) and in Y79 human retinoblastoma cells (Kyritsis et al., 1987b; Yorek et al., 1987; Pierce et al., 1989; Janavs et al., 1991).

In Y79 retinoblastoma cells, increases in NAT activity or melatonin production depend on an elevation in cyclic AMP (cAMP), through a mechanism involving protein synthesis (Pierce et al., 1989; Janavs et al., 1991). We have recently demonstrated that in Y79 cells the RNA synthesis inhibitors camptothecin and actinomycin D *increase* melatonin levels (Janavs et al., 1994). Camptothecin also increases NAT activity and cAMP levels in a calcium-dependent manner. Combined treatment with camptothecin and forskolin causes a synergistic elevation of melatonin production.

To determine which proteins might be affected by the action of forskolin and camptothecin, we have compared the two-dimensional protein profiles from untreated Y79 cells with those from Y79 cells treated with these agents. The radiolabeling of a cytosolic protein of $M_r \approx 30$ kDa, pI ≈ 5.7 (p30) was consistently increased in parallel with the increases in NAT activity and melatonin induced by these treatments. We propose that p30 may be either a component or a regulator of the melatonin synthesis pathway.

Materials and Methods

Y79 retinoblastoma cell maintenance and general experimental procedures are described in detail in Janavs et al. (1991). Briefly, Y79 retinoblastoma cells (Reid et al., 1974; Kyritsis et al., 1987a) (American Type Tissue Culture, Rockville, MD) were maintained in suspension culture on a 12 hr light/12 hr dark schedule at 37°C (5% CO_2) in Dulbecco's modified Eagle's media (DME media with glutamine and glucose; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin (GIBCO), referred to as DME complete medium. For experiments, cells were plated at 5×10^5 or 1×10^6 cells/ml in 24-well culture dishes (Costar, Cambridge, MA) previously coated with poly-*d*-lysine (MW 150,000–300,000; Sigma Chemical Co., St. Louis, MO). Cells were allowed to attach for 24 hr.

For experiments, the medium was exchanged for 0.5 ml of experimental medium consisting of DME complete plus 100 μM 5-hydroxytryptophan (5-HTP; Sigma), without (basal) or with (stimulated) 10 μM forskolin and/or the RNA synthesis inhibitor camptothecin at 10^{-6} gm/ml. 5-HTP is added to the media so that serotonin availability is not rate limiting (Pierce et al., 1989). The protocols for individual gel experiments were as follows.

The effect of 6 hr camptothecin treatment on protein synthesis. In two experiments, triplicate wells of Y79 cells were treated for 6 hr with low methionine DME (GIBCO) complete media plus 100 μM 5-HTP (Sigma) and 100 μCi/ml ^{35}S-methionine alone (basal), or with 10 μM forskolin only (fsk), with 10^{-6} gm/ml camptothecin only (camptothecin), or 10 μM forskolin and 10^{-6} gm/ml camptothecin together (fsk + camptothecin). The cells were then prepared as two-dimensional gel samples, as described below, boiled a second time for 3 min, diluted 2:1 in solution E [0.1 gm dithiothreitol/0.4 gm CHAPS (Bio-Rad, Richmond, CA)/5.4 gm urea/500 μl Bio-Lyte 3/10 ampholyte in 6 ml of ddH_2O], and 15 μl (~20 μg protein) loaded on isoelectric focusing (IEF) gels. Aliquots of the original samples were TCA precipitated to quantify effects on total protein synthesis (described in detail in Janavs et al., 1991).

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Correspondence should be addressed to Joseph S. Takahashi, NSF Center for Biological Timing, Department of Neurobiology and Physiology, Northwestern University, 2153 North Campus Drive, Evanston, IL 60208-3520.

[a] Present address: University of California at San Francisco, Department of Medicine, San Francisco, CA 94143-0114.

[b] Present address: SUNY Health Sciences Center at Syracuse, Department of Physiology, Syracuse, NY 13210-2339.

Table 1. Proteins altered by 6 hr treatment with forskolin and camptothecin

Protein	MW (kDa)	pI	Basal	Camptothecin	Forskolin	Forskolin + camptothecin
p30	30	5.7	+	++	+++	++++
p28	28	5.9–6.3	+++	++++	+	+
p29	29	5.6	0	±	++	++
p35	35	4.0–4.6	0	0	++	++
p45A	45	5.8–5.9	+	++	+	++
p45B	45	6.0–6.2	+	++	+	++
p50	50	5.8–5.9	+	++	+	++

Triplicate wells of Y79 cells were plated at 10^6 cells/well and treated for 6 hr with 100 μCi/ml ^{35}S-methionine and either 10^{-6} gm/ml camptothecin, 10 μM forskolin, or 10^{-6} gm/ml camptothecin plus 10 μM forskolin in low methionine media. Protein samples were prepared and run on two-dimensional gels followed by autoradiography as described in Materials and Methods. 0 = no radiolabeling; + = increases in protein labeling; ± = equivocal increase in radiolabeling in arbitrary units.

Time course of the effects of camptothecin on protein synthesis. Triplicate wells of Y79 cells were pulse labeled for 1 hr with 200 μCi/ml ^{35}S-methionine during the first, second, third, and sixth hour of incubation in low methionine DME complete media and 100 μM 5-HTP alone, or with 10 μM forskolin, or 10^{-6} gm/ml camptothecin, or 10 μM forskolin and 10 gm/ml camptothecin together. Cell plating, treatment, and sampling for the entire time course were carried out together in one experiment. Two-dimensional gel samples were prepared as described below, boiled a second time for 3 min, diluted 2:1 in solution E, and 25 μl (~33 μg of protein) loaded on IEF gels. The two-dimensional gels were run in sets of 12, each set consisting of one pulse time (e.g., first hour pulse, second hour pulse, etc.). Effects on total protein synthesis were assayed by TCA protein precipitation.

Pulse–chase. Triplicate wells of experimental Y79 cells were labeled for 3 hr with 100 μCi/ml ^{35}S-methionine in low methionine DME complete, washed with Hank's Balanced Salt Solution (HBSS; GIBCO), and incubated for 3 more hr with unlabeled methionine DME complete and 100 μM 5-HTP alone (pulse control + chase control) or with 10 μM forskolin plus 10^{-6} gm/ml camptothecin (pulse control + chase F+C). For controls, triplicate wells of Y79 cells were incubated for 3 hr in DME complete, washed with HBSS, and incubated for the second 3 hr in 100 μCi/ml ^{35}S-methionine in low methionine DME complete and 100 μM 5-HTP alone (pulse control) or with 10 μM forskolin plus 10^{-6} gm/ml camptothecin (pulse F+C). Two-dimensional gel samples were prepared as described below, boiled a second time for 3 min, diluted 2:1 in solution E, and 15 μl loaded on IEF gels. Effects on total protein synthesis were assayed by TCA protein precipitation. The experiment was carried out twice.

Cell fractionation. Y79 cells were plated in duplicate at 4 × 10^6 cells/35 mm well for 24 hr and labeled for 6 hr with 2 ml of DME containing 100 μCi/ml of ^{35}S-methionine, in the absence and presence of 10 μM forskolin and 10^{-6} gm/ml camptothecin. The cells were then washed with cold HBSS, scraped into isotonic homogenization buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1 mM EDTA, pH 7.4) and homogenized on ice with ~12 strokes of a Dounce glass homogenizer using a type B pestle. The homogenate was centrifuged at 1000 × *g* for 10 min and the nuclear pellet resuspended in homogenization buffer and frozen on dry ice. The supernatant was centrifuged at 40,000 × *g* for 60 min, the particulate pellet resuspended in homogenization buffer, and frozen on dry ice together with the cytosolic fraction. All centrifugation fractions were evaluated by phase-contrast microscopy.

An equal volume of 2× sample buffer was added to each sample for gel electrophoresis and incorporated counts were assayed by TCA precipitation. Duplicate samples normalized to TCA precipitable counts were loaded on two-dimensional gels and electrophoresed as described below. Gels were dried and exposed to X-OMAT autoradiography film for 29 d.

Chemicals. Forskolin was obtained from Calbiochem Corp. (La Jolla, CA), radiochemicals were from Amersham (Arlington Heights, IL), gel components were from Bio-Rad (Richmond, CA), and all other drugs were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

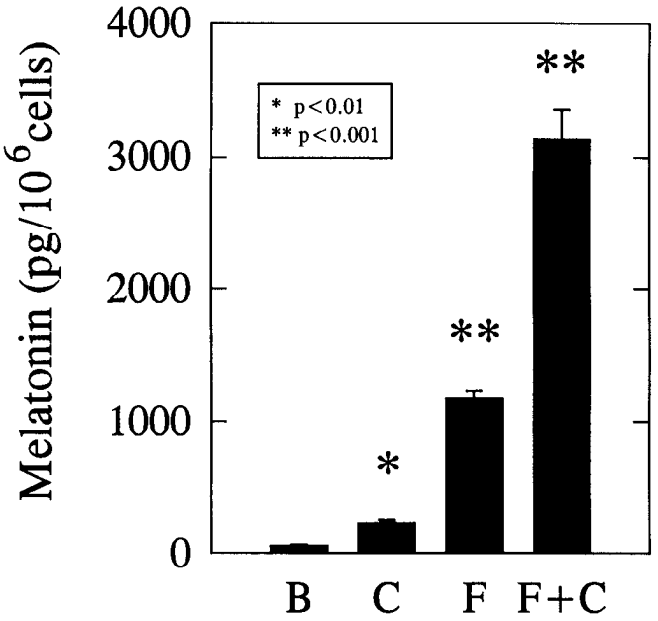

Figure 1. Camptothecin and forskolin, alone and in combination, significantly increase the production of melatonin by Y79 human retinoblastoma cells. *B*, basal; *C*, 10^{-6} gm/ml camptothecin; *F*, 10 μM forskolin; and *F+C*, forskolin plus camptothecin.

Table 2. Effects of forskolin and camptothecin on p30 radiolabeling intensity

	Protein database units × 10^7 (mean ± SEM)					
	p30	p27	p36	p31	p32	p40
Control	23.0 ± 23.0*	79.7 ± 5.8	104.0 ± 13.3	54.7 ± 8.4	121.0 ± 12.0	86.3 ± 2.4
Camptothecin	116.3 ± 30.8	103.7 ± 16.9	115.3 ± 15.0	61.3 ± 5.2	147.0 ± 12.1	112.3 ± 12.0
Forskolin	125.7 ± 11.3	88.3 ± 11.8	122.0 ± 11.6	53.0 ± 2.5	136.0 ± 11.3	108.7 ± 10.2
Forskolin + camptothecin	228.3 ± 21.5*	114.0 ± 7.5	118.0 ± 5.0	69.7 ± 2.4	157.0 ± 7.0	142.0 ± 8.1*

Triplicate wells of Y79 cells were plated at 10^6 cells/well and treated for 6 hr with 100 μCi/ml ^{35}S-methionine and either 10^{-6} gm/ml camptothecin, 10 μM forskolin, or 10 μM forskolin plus 10^{-6} gm/ml camptothecin in low methionine media. Protein samples were prepared and run on two-dimensional gels followed by autoradiography as described in Materials and Methods, and radiolabeling was quantified using PDQUEST. Radiolabeling of protein p30 is increased above control levels by all three drug treatments. The other proteins listed (p27, p36, p31, p32, and p40) are controls that do not vary significantly between treatment groups. Protein database units are arbitrary relative units used by PDQUEST.

* Means for these treatment groups are significantly different ($p < 0.05$) from any other group for a given protein (Duncan's post hoc test); means of unmarked groups are not significantly different from each other for a given protein.

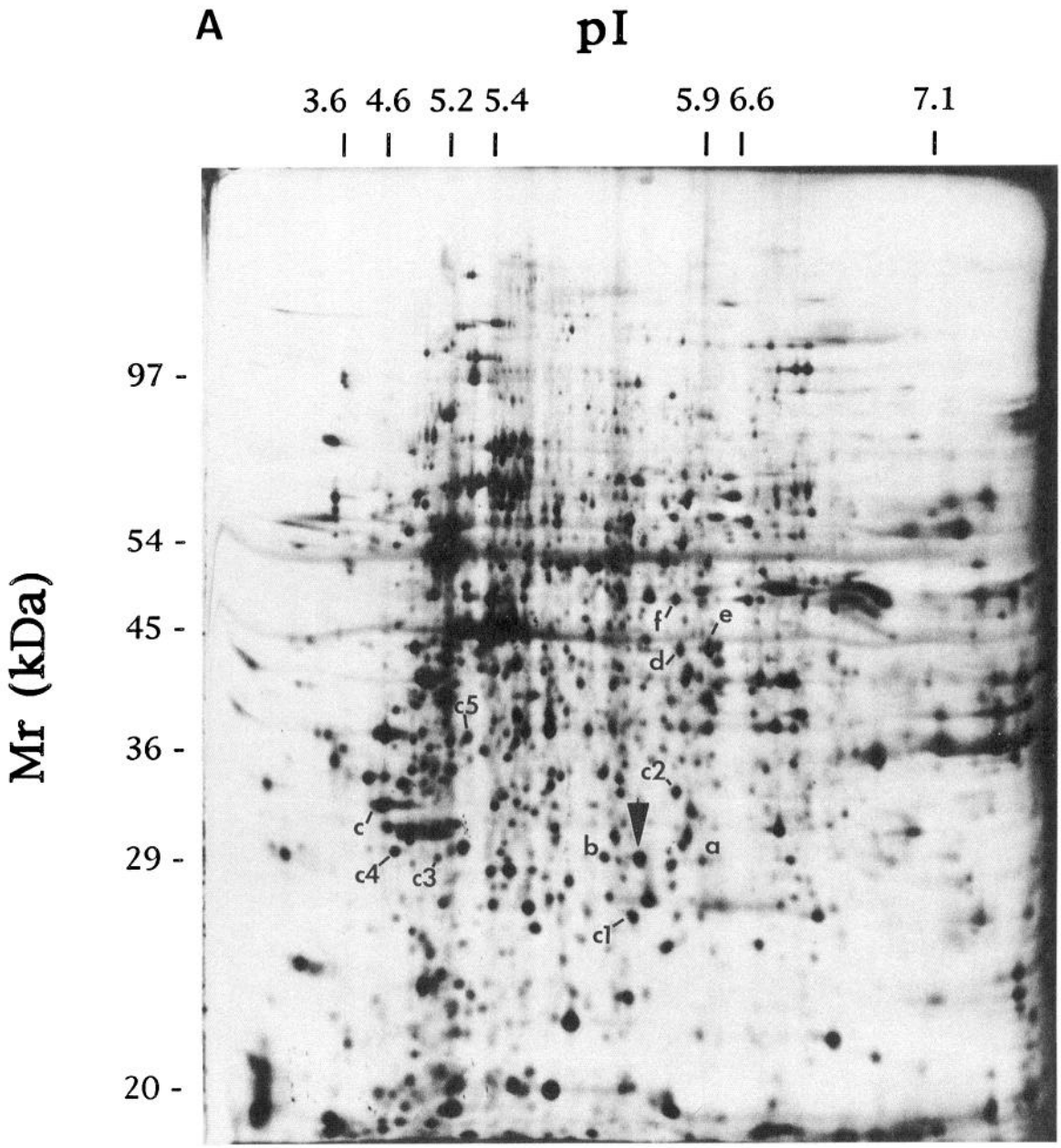

Figure 2. *A,* Representative two-dimensional autoradiograph of ^{35}S-methionine–labeled proteins from forskolin plus camptothecin–stimulated Y79 cells. Y79 cells were plated at 10^6 cells/well and treated for 6 hr with 100 μCi/ml ^{35}S-methionine and either 10^{-6} gm/ml camptothecin, 10 μM forskolin, or 10 μM forskolin plus 10^{-6} gm/ml camptothecin in low methionine DME complete, as described in Materials and Methods. Proteins whose radiolabeling is altered by these treatments are indicated by *letters* (*arrow* = p30, *a* = p28, *b* = p29, *c* = p35, *d* = p45A, *e* = p45B, *f* = p50); control proteins are indicated by the *letter c* followed by a *number* (*c1* = p27, *c2* = p36, *c3* = p31, *c4* = p32, and *c5* = p40). *B,* Magnified composite of the area containing protein p30, from two-dimensional gels obtained from basal, camptothecin, forskolin, and forskolin plus camptothecin–stimulated Y79 cells. The radiolabeling of protein p30 (*arrowhead*) increases in parallel with melatonin production in the three drug treatments. Landmark proteins are *circled.*

Melatonin radioimmunoassay. Melatonin levels in medium were assayed by the method of Rollag and Niswender (1976), as modified by Takahashi et al. (1980). This assay has been validated for Y79 cells (Pierce et al., 1989), and has a limit of sensitivity of 0.5 pg of melatonin per tube.

Sample preparation for two-dimensional analytical gels. Samples were prepared using a modification of the protocol of Garrels (1983). Briefly, treated Y79 cells were rinsed with cold HBSS and solubilized and scraped in 100 μl/well sodium dodecyl sulfate–β-mercaptoethanol buffer (SDSBME; 0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol), and 10 μl/well 10× nuclease mix (1 mg/ml DNase I, 0.5 mg/ml RNase A, 50 mM $MgCl_2$, 0.5 M Tris-HCl, pH 7). These lysates were rapidly transferred to microfuge tubes, boiled for 3 min, frozen in dry ice/ethanol, and stored at −70°C until electrophoresis.

Two-dimensional analytical gels. Two-dimensional analytical gel electrophoresis and autoradiography were carried out with minor modifications, according to the protocol of Hochstrasser et al. (1988). Proteins in equal volumes of samples were first separated on isoelectric focusing gels using a 1:4 mixture of pH 5–7 and pH 3–10 ampholines, and run at 200 V for 2 hr, 500 V for 5 hr, 800 V for 14 hr, and 990 V for 2 hr. The IEF gels were then overlaid onto 1.5 mm thick 10% SDS-polyacrylamide slab gels for separation of the proteins by molecular weight. Gels were calibrated with protein markers of known molecular weight and pI (amyloglucosidase, 97 kDa, pI 3.6, cat. A2910; carbonic

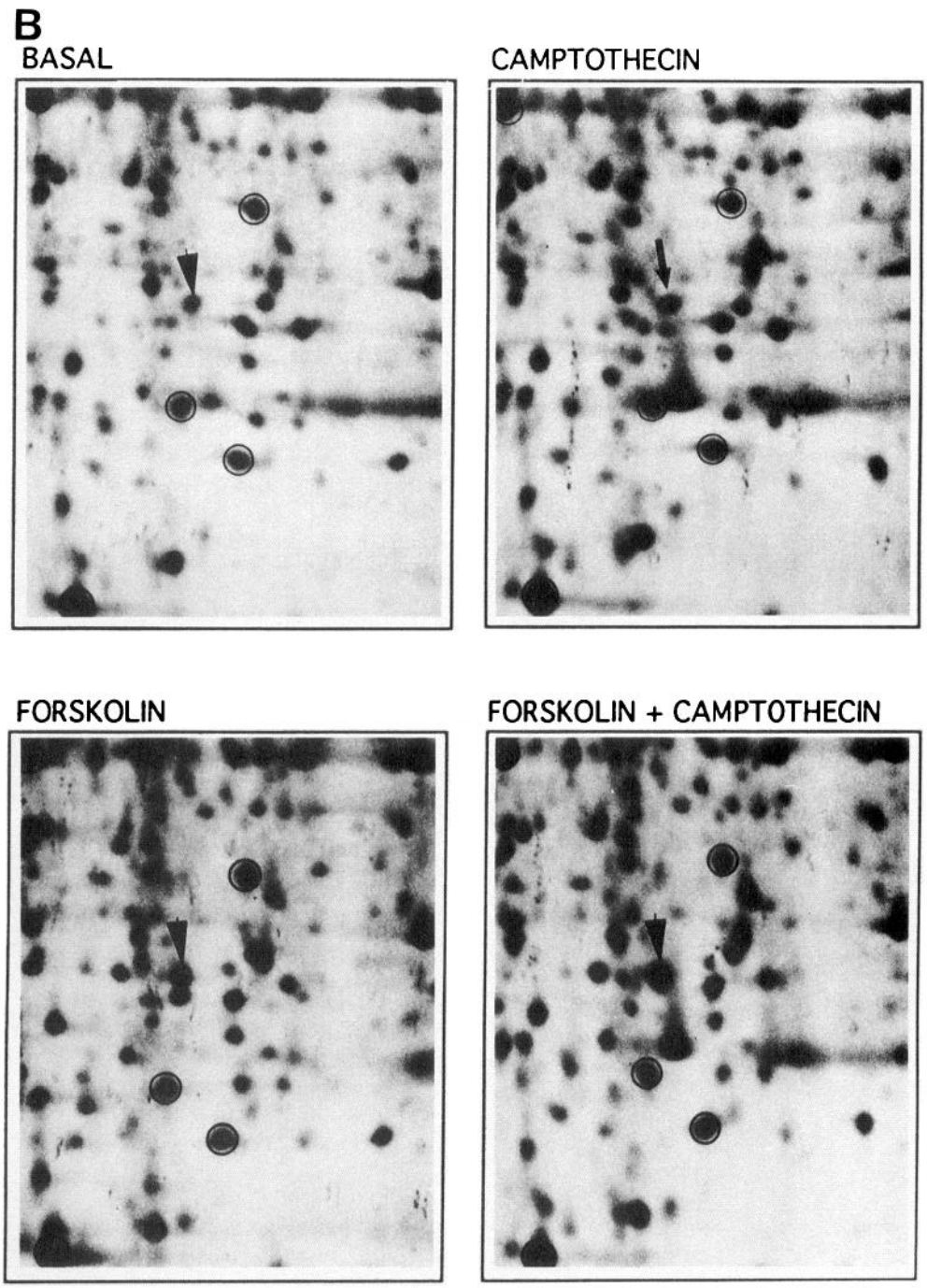

Figure 2. Continued.

anhydrase I, 29 kDa, pI 6.6, cat. C6653; carbonic anhydrase II, 29 kDa, pI 5.9, cat. C6403; L-lactic dehydrogenase, 36.5 kDa, pI 8.3, 8.4, 8.6, cat. L5012; β-lactoglobulin A, 18.4 kDa, pI 5.1, cat. L5137; hemoglobin A, 64.5 kDa, pI 7.1, cat. H0267; myoglobin, 17.5 kDa, pI 6.8, 7.2, cat. M9267; trypsin inhibitor, 20.1 kDa, pI 4.6, cat. T1021; Sigma). The slab gels were fixed in 10% glacial acetic acid/20% methanol overnight, dried onto filter paper, and exposed to Kodak X-AR-OMAT film for 1 and 5 d. Labeling of proteins of interest was quantified using PDQUEST two-dimensional gel analysis software from Protein Databases Inc. Proteins whose radiolabeling was minimally altered by the drug treatments were used as controls (p27, p36, p31, and p32-tropomyosins, and p40).

Gel staining. For Coomassie staining, gels were incubated with shaking in 0.2% Coomassie blue G (w/v)/9% acetic acid/46% methanol for 30 min, destained for 2 hr in several changes of 10% acetic acid/20% methanol, and dried.

Silver staining was carried out according to the protocol of Hochstrasser et al. (1988). Briefly, gels were incubated for 1 hr in 10% glacial acetic acid/40% ethanol fix and then overnight in 5% glacial acetic acid/5% ethanol with shaking. They were then soaked for 30 min in 2.5% glutaraldehyde and washed extensively (3 × 10 min, 4 × 30 min) in cold ddH_2O. Staining (0.5% ammonium hydroxide/0.02 M NaOH/47.1 mM silver nitrate) was carried out for 30 min followed by 3 × 5 min of washing in ddH_2O. Gels were developed for 1–3 min (0.1% formaldehyde/0.34 mM citric acid), stopped with 5% glacial acetic acid for 15 min, and stored in 7% glycerol/10% glacial acetic acid until dried.

Immunoblotting. Two-dimensional electrophoresis was performed as

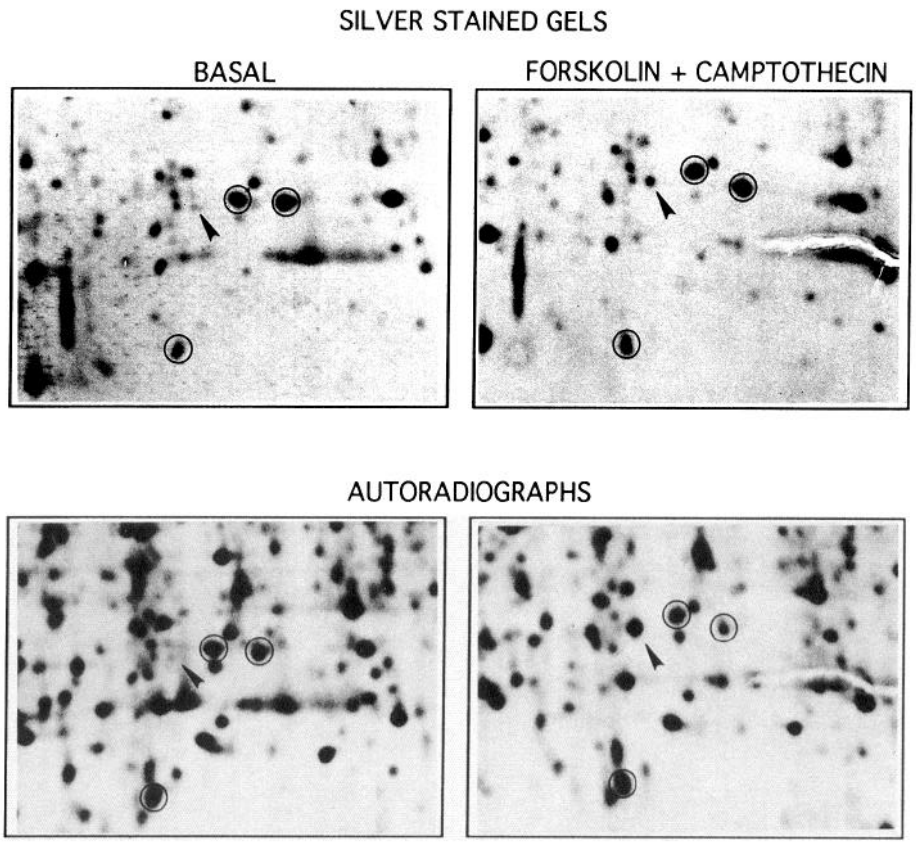

Figure 3. Photographs of two-dimensional gel electrophoretic patterns of silver-stained proteins (*top*) and associated autoradiographs (*bottom*) from basal (*left*) and forskolin plus camptothecin–stimulated (*right*) Y79 cells. Y79 cells were treated as described in Figure 2 and Materials and Methods. Protein p30 (*arrowhead*) is faintly visible in basal samples and increases in forskolin plus camptothecin–stimulated Y79 cell protein samples. Landmark proteins are *circled*. Gels are representative of two experiments.

above. Relevant portions of the gels were cut and incubated in blotting buffer (25 mM Tris, 192 mM glycine, and 15% methanol, pH 8.3) for 30 min. Transfer onto PVDF membranes (Immobilon P; Millipore) was carried out at 200 mA for 1 hr in a Hoefer Mini-Transphor apparatus. Immunodetection was performed with the ECL kit (Amersham) using the manufacturer's protocol. The antibodies were diluted in Tris-buffered saline with 0.1% Tween (TTBS) at 1:10,000 for anti-14-3-3 (Roseboom et al., in press), 1:10,000 for anti-cytosolic malate dehydrogenase (cMD, Felder, 1978), and 1:1,000 for anti-recoverin (Dizhoor et al., 1991). Blots were incubated in this solution for 1, 3, and 18 hr, respectively. Because the antibody against recoverin cross-reacts with other proteins (Korf et al., 1992), the blot was stripped with stripping buffer (100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris, pH 6.7) for 30 min at 50°C and reprobed with a 1:1000 dilution of recoverin antibody that had been preabsorbed with 17 μg/ml recombinant bovine recoverin overnight.

Statistical analysis. Radiolabeling intensity (cpm) of proteins was normalized to the TCA-precipitable ^{35}S-methionine counts for each sample. Statistical significance between groups of replicates was determined by one-way analysis of variance and Duncan's post hoc test (SAS Institute Inc.).

Table 3. Time course of the effects of forskolin and camptothecin on p30 radiolabeling intensity

	Protein database units $\times 10^7$ (mean ± SEM)			
	1st	2nd	3rd	6th hr pulsed
Control	41.3 ± 29.3*	44.0 ± 25.4	89.0 ± 14.6	0 ± 0*
Camptothecin	112.7 ± 10.7*	134.3 ± 45.7‡	70.3 ± 0.9§	182.3 ± 15.6*
Forskolin	328.7 ± 36.5*	223.0 ± 36.4†	230.0 ± 48.2*	222.0 ± 10.6*
Forskolin + camptothecin	270.7 ± 32.4*	382.3 ± 24.3*	419.3 ± 62.1*	606.5 ± 1.5*#

Y79 cells were plated in triplicate at 10^6 cells/well and pulsed for 1 hr with 200 μCi/ml ^{35}S-methionine during the first, second, third, and sixth hour of incubation in low methionine DME complete media alone, and either 10^{-6} gm/ml camptothecin, 10 μM forskolin, or 10 μM forskolin plus 10^{-6} gm/ml camptothecin. Radiolabeling was quantified using PDQUEST. Values have been normalized to TCA-precipitable ^{35}S-methionine levels. Protein p30 labeling is present in all four conditions after the first hour of incubation and does not change significantly with an increasing duration of drug treatment. The radiolabeling of four control proteins was minimally or inconsistently altered by the treatments (see Janavs, 1992).

* Means for these groups are significantly different from any other group for a given pulse hour ($p < 0.05$).

† Mean is significantly different from all other groups except camptothecin in the 2nd hour.

‡ Camptothecin is not significantly different from control or forskolin in the 2nd hour.

§ Camptothecin is significantly different from all groups except control in the 3rd hour (Duncan's post hoc test).

Only duplicate samples were available for this group.

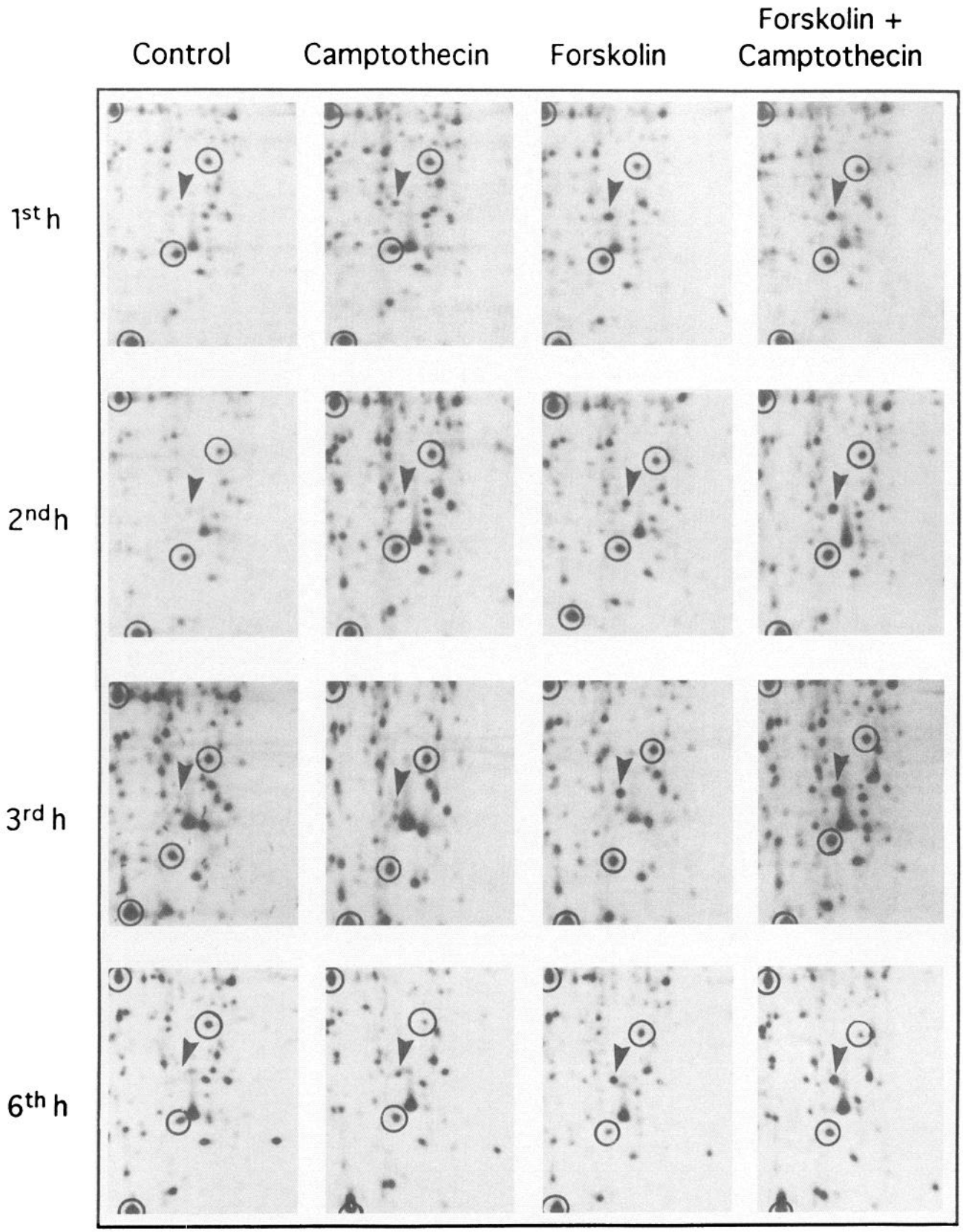

Figure 4. Time course of effects of forskolin and camptothecin on the individual proteins in Y79 cells. Y79 cells were plated at 10^6 cells/well and pulsed for 1 hr with 200 μCi/ml ^{35}S-methionine during the first, second, third, and sixth hour of incubation in low methionine DME complete media alone, or with 10 μM forskolin, or 10^{-6} gm/ml camptothecin, or 10 μM forskolin plus 10^{-6} gm/ml camptothecin. Protein p30 (*arrowhead*) is present in all four conditions after the first hour of incubation and generally increases in radiolabeling intensity with increasing duration of drug treatment. Landmark proteins are *circled*. Gels are representative of three replicates per treatment from one experiment.

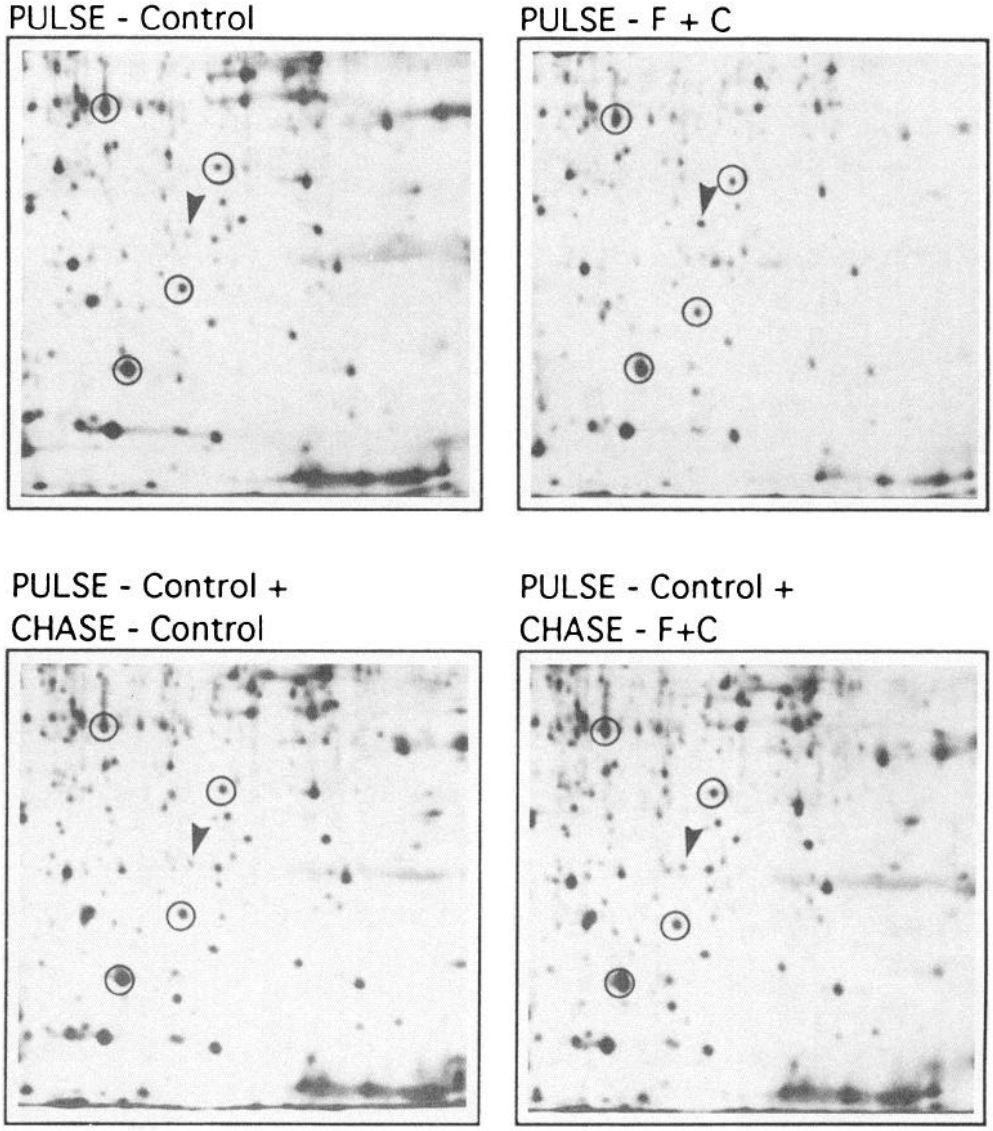

Figure 5. Pulse–chase labeling of control and forskolin plus camptothecin–treated Y79 cells. Y79 cells were plated at 10^6 cells/well. Control Y79 cells were left untreated for 3 hr and then incubated in 100 μCi/ml ^{35}S-methionine in the absence (*PULSE – Control*) and presence of 10 μM forskolin plus 10^{-6} gm/ml camptothecin (*PULSE – F + C*) for 3 hr. Experimental Y79 cells were first pulsed for 3 hr with 100 μCi/ml ^{35}S-methionine and then chased for 3 hr in cold methionine in the absence (*PULSE – Control + CHASE – Control*) and presence of 10 μM forskolin plus 10^{-6} gm/ml camptothecin (*PULSE – Control + CHASE – F + C*). The radiolabeling of protein p30 (*arrowhead*) is increased by F+C over control levels (*top panels*). In cells labeled with ^{35}S-methionine, subsequent exposure to F+C in the presence of cold methionine produced no change in the amount of radiolabeled p30. In all six replicates of both experiments the protein radiolabeling pattern was consistently different in the pulse F+C gels, although p30 was still easily identifiable. Gels are representative of three replicates per treatment from two experiments.

Results

Melatonin synthesis

Y79 human retinoblastoma cells synthesize detectable amounts of melatonin when incubated in the presence of melatonin precursor 5-HTP. Six hour treatments of Y79 cells with 10^{-6} gm/ml camptothecin only, 10 μM forskolin only, or 10^{-6} gm/ml camptothecin and 10 μM forskolin together, significantly increased the amount of melatonin synthesized (Fig. 1).

Table 4. Protein p30 labeling during pulse-chase

	Protein database units $\times 10^7$ (mean ± SEM)			
	Pulse control	Pulse F + C	Pulse control + chase control	Pulse control + chase F + C
Experiment 1	43.5 ± 2.5	240.0 ± 83.0*	65.7 ± 24.8	74.3 ± 5.0
Experiment 2	70.5 ± 16.5	390.0 ± 4.0*	89.0 ± 5.0	121.7 ± 30.1

Y79 cells were plated at 10^6 cells/well and labeled for 3 hr with 100 μCi/ml ^{35}S-methionine in the absence (pulse control) and presence of 10 μM forskolin plus 10^{-6} gm/ml camptothecin (pulse F + C). Control Y79 cells initially treated for 3 hr with 100 μCi/ml ^{35}S-methionine were then incubated in cold methionine in the absence (pulse control + chase control) and presence (pulse control + chase F + C) of 10 μM forskolin plus 10^{-6} gm/ml camptothecin. $N = 2$ for pulse groups; $N = 3$ for pulse–chase groups. Radiolabeling was quantified using PDQUEST. Values have been normalized to TCA-precipitable ^{35}S-methionine levels. The radiolabeling intensity of protein p30 in the pulse–chase groups is similar in intensity to that in the pulse control group but is increased in the pulse F + C group.

* Mean for this group is significantly different from any other group ($p < 0.05$, Duncan's post hoc test). The radiolabeling of four control proteins was minimally or inconsistently altered by the treatments (see Janavs, 1992).

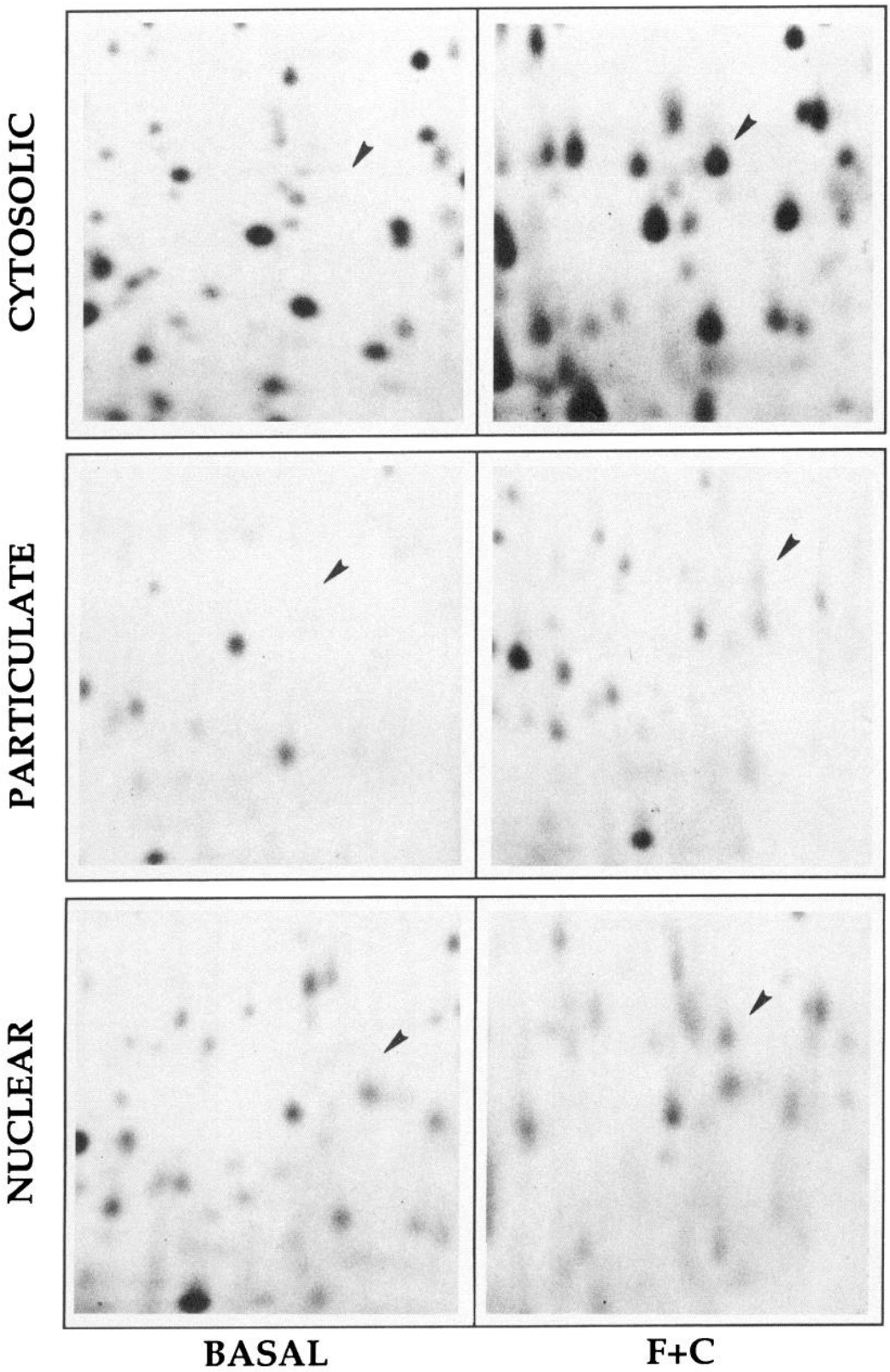

Figure 6. Protein p30 is located in the cytosol. Y79 cells plated at 4×10^6 cells/35 mm well were treated for 6 hr with 100 μCi/ml ^{35}S-methionine in the absence and presence of 10 μM forskolin plus 10^{-6} gm/ml camptothecin. The cells were then fractionated and electrophoresed in two dimensions. Protein p30 labeling (*arrowhead*) is clearly increased in the cytosolic fraction of forskolin plus camptothecin–stimulated cells compared to basal levels, but not in the particulate or nuclear fractions.

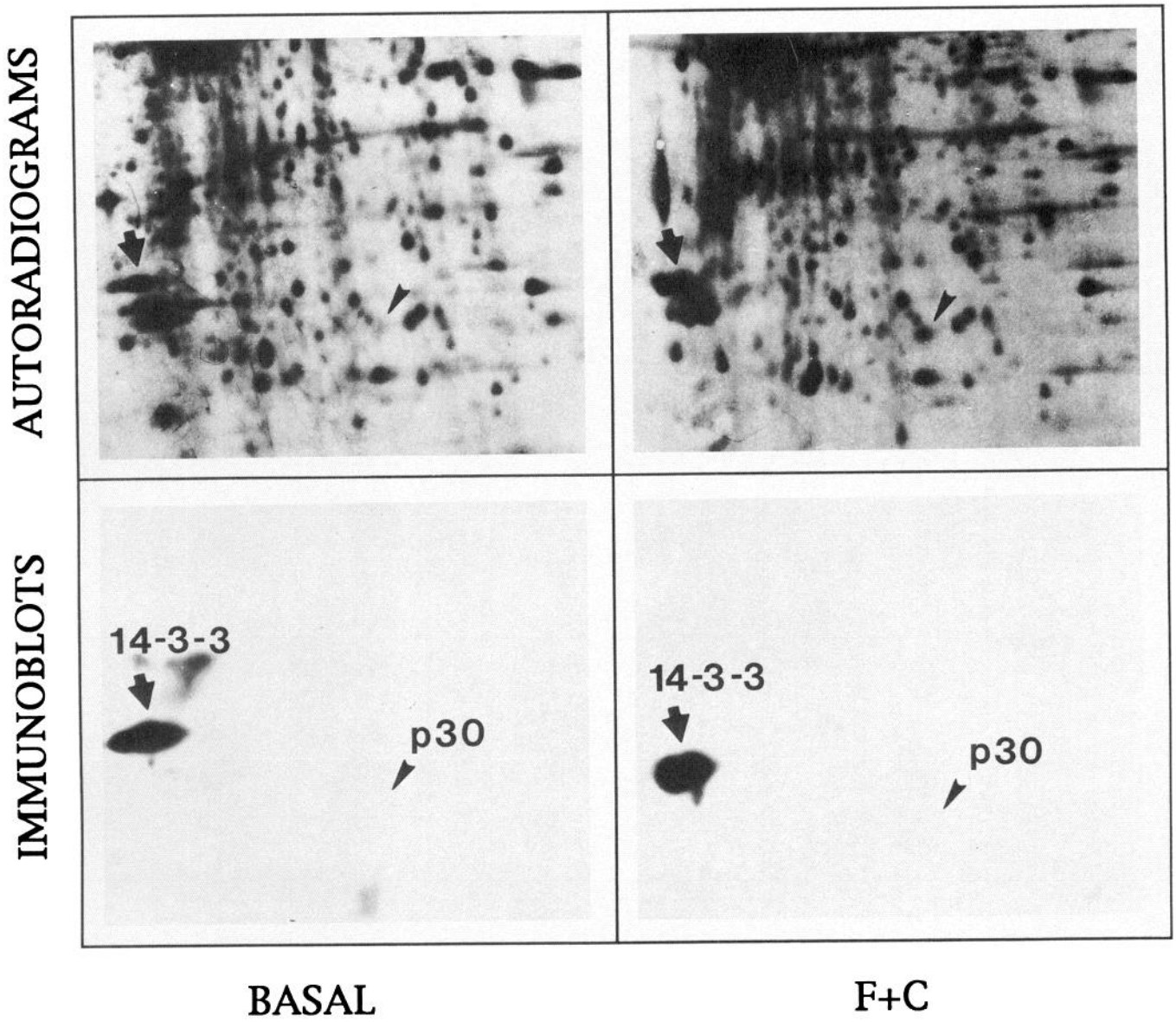

Figure 7. Two-dimensional immunoblots of basal (*left*) and forskolin plus camptothecin–stimulated (*right*) Y79 cells, probed with a monoclonal antibody against the 33 kDa isoform of protein 14-3-3. Cells were radioactively labeled with ^{35}S-methionine, loaded on two-dimensional gels, and blotted onto PVDF membranes. After immunodetection (*bottom*), the blots were dried and exposed for autoradiography (*top*). *Arrow,* protein 14-3-3; *arrowhead,* p30. Protein p30 has a lower molecular weight and more basic isoelectric point than protein 14-3-3 in Y79 human retinoblastoma cells.

Six hour treatment

Two-dimensional gel analysis of forskolin- and/or camptothecin-treated Y79 cells was performed to identify proteins that changed in parallel with the melatonin response. Relative to controls, Y79 retinoblastoma cells treated continuously for 6 hr with forskolin, camptothecin, or forskolin plus camptothecin showed no significant change in overall protein synthesis measured as TCA-precipitable counts (data not shown). However, on two-dimensional gels, several proteins were identified that consistently changed with the various drug treatments (Table 1, Fig. 2); their relative positions are shown on representative gels in Figure 2*A*. Forskolin treatment increased radiolabeling of three individual proteins (p29, p30, and p35), and decreased the labeling of one protein (p28) relative to controls. Camptothecin treatment increased the labeling of five proteins (p28, p30, p45A, p45B, and p50). Simultaneous treatment with both agents increased labeling of six proteins (p29, p30, p35, p45A, p45B, and p50). Of the eight proteins observed to change with drug stimulation, only p30 ($M_r \approx 30$ kDa, pI ≈ 5.7) increased consistently in parallel with the melatonin response in all four treatments (Fig. 2*B*); thus, we have focused our analysis on this protein. Forskolin or camptothecin treatment alone increased radiolabeling of p30 fivefold, while combined drug treatment increased labeling 10-fold (Table 2). For untreated cells, protein p30 was not visible by Coomassie staining, and was barely detectable in silver stained control gels, but was clearly present in silver-stained forskolin plus camptothecin–treated samples (Fig. 3).

Time course of effects on protein synthesis

To determine how rapidly p30 levels changed after drug treatment, Y79 cells were pulse labeled for 1 hr during the first, second, third, and sixth hour of incubation in drug-containing media. The radiolabeling of protein p30 was increased four- to eightfold by camptothecin and forskolin, alone or in combination, during the first hour of incubation (Fig. 4, Table 3). Thereafter, p30 radiolabeling showed no further increase but remained high throughout the next 6 hr. This suggests that p30 is induced rapidly and near maximally within the first hour of incubation. Although other proteins also exhibited changes in radiolabeling intensity, they did not do so in a melatonin-related manner.

Pulse–chase

In the 6 hr and time-course experiments we observed that forskolin and camptothecin increased the radiolabeling of protein p30. Using a pulse–chase design we examined whether the observed rise of p30 radiolabeling was due to increasing its synthesis or inhibiting its degradation. Y79 cells were incubated in ^{35}S-methionine (pulse) to label radioactively the proteins being synthesized in the basal state. After labeling, the unincorporated extracellular ^{35}S-methionine was washed out and replaced with media lacking ^{35}S-methionine, but containing forskolin and camptothecin (chase). If forskolin and camptothecin stimulated the synthesis of p30, their addition in the absence of ^{35}S-methionine during the chase should increase the synthesis of unlabeled p30 (pulse control + chase F+C). This should have no effect on the radiolabeling intensity of p30 relative to control samples treated with ^{35}S-methionine under basal conditions (pulse control) for the same length of time. If forskolin and camptothecin inhibited the degradation of p30, then the addition of these drugs in the absence of ^{35}S-methionine during the chase would decrease the degradation of labeled p30, resulting in levels of radiolabeled p30 similar to those in samples treated with ^{35}S-methionine, forskolin, and camptothecin (pulse F+C) for the same length of time, but greater than control samples treated with ^{35}S-methionine under basal conditions (pulse control).

Two pulse–chase experiments were carried out and the radiolabeling intensity of p30 was normalized to the TCA-precipitable ^{35}S-methionine counts for each sample (Fig. 5, Table 4). Consistent with our previous results, the pulse control samples demonstrated basal levels of protein p30 radiolabeling and the pulse F+C gels showed a significant increase (five- to sixfold) in the radiolabeling of p30. In the pulse control + chase control samples and the pulse control + chase F+C gels, p30 labeling was indistinguishable from pulse control levels, suggesting that both are at basal levels. If forskolin and camptothecin were inhibiting p30 degradation, radiolabeled cells chased with F+C should have had higher levels of labeled p30 than radiolabeled cells chased with drug-free media (the labeled protein in the cells receiving a drug-free chase would have continued to degrade at its normal rate). As this was not the case in our experiments, a degradative mechanism is not supported. Although it might be argued that 3 hr is too brief a time in which to observe inhibitory effects on p30 degradation, we are able to see forskolin and camptothecin–stimulated increases in p30 radiolabeling in 3 hr. Thus, our results are consistent with the hypothesis that forskolin and camptothecin stimulate p30 synthesis.

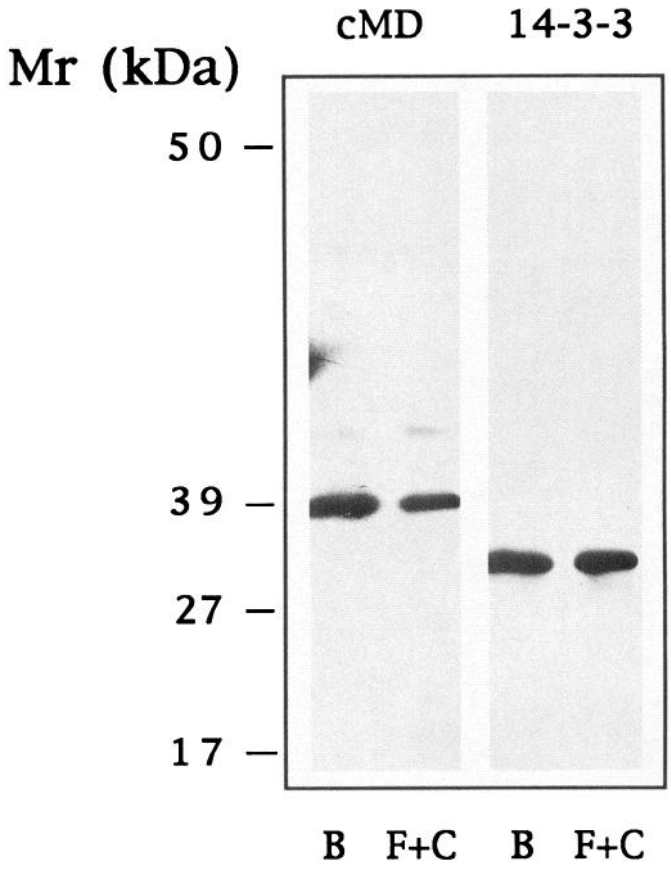

Figure 8. One-dimensional immunoblots of basal (*B*) and forskolin plus camptothecin–stimulated (F+C) Y79 cells, probed with antibodies against cytosolic malate dehydrogenase (cMD) and protein 14-3-3. Malate dehydrogenase has a higher molecular weight than 14-3-3 in Y79 human retinoblastoma cells, making it an unsuitable candidate for p30.

Cell fractionation studies. To determine the cellular location of protein p30 we compared the two-dimensional protein profiles of basal and forskolin plus camptothecin–stimulated nuclear, particulate, and cytosolic fractions from Y79 cells (Fig. 6). Protein p30 was clearly present in the cytosolic fraction of cells stimulated with forskolin and camptothecin, but only trace amounts, which were presumed to be contaminants, were detectable in the nuclear and particulate fractions.

Immunoblotting

Proteins with characteristics similar to those of p30 include the 14-3-3 protein family, the cytosolic form of malate dehydrogenase, recoverin, and arylalkylamine-NAT. A two-dimensional immunoblot with monoclonal antibody 8C3 raised against the 33 kDa isoform of protein 14-3-3 showed that p30 and 14-3-3 are different proteins, with p30 having a lower molecular weight and more basic isoelectric point (Fig. 7). These results were confirmed with another anti-14-3-3 monoclonal antibody, 5G1 (data not shown). The anti-14-3-3 antibody cross-reacts with p35, a protein that is slightly more acidic than the major 14-3-3 isoform and whose radiolabeling is increased by forskolin (Table 1). It is possible that p35 may be a phosphorylated form of 14-3-3, though this possibility is as yet untested. A one-dimensional immunoblot comparing protein 14-3-3 and cytosolic malate dehydrogenase showed that in human Y79 reti-

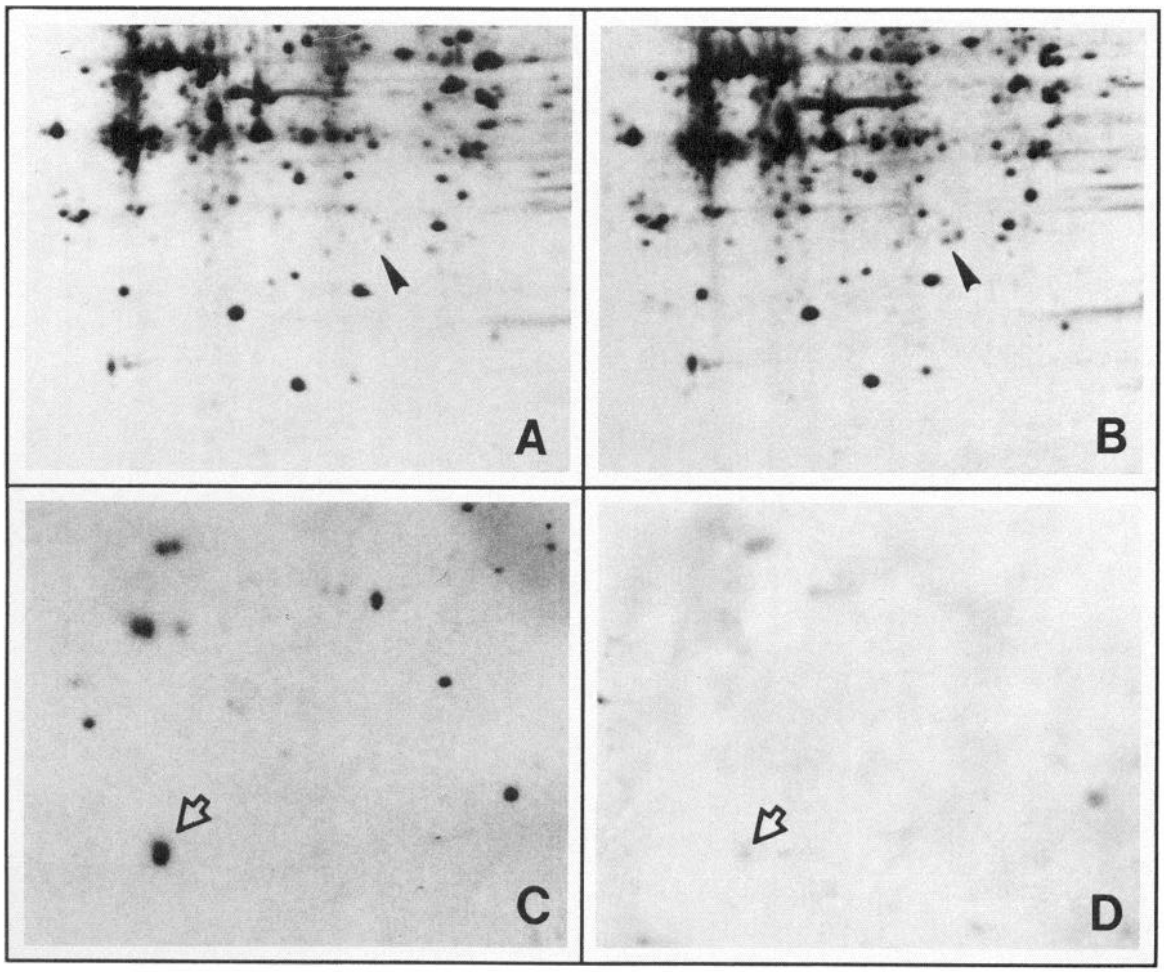

Figure 9. Two-dimensional immunoblots of forskolin plus camptothecin–stimulated Y79 cells, prepared as in Figure 7 and probed with an antibody against bovine recoverin. *A*, autoradiogram, basal conditions; *B*, autoradiogram, forskolin + camptothecin; *C*, immunoblot of stimulated cells probed with the anti-recoverin antibody; *D*, same blot as *C*, stripped and reprobed with an antibody solution that had been preabsorbed with 17 μg/ml of recombinant recoverin overnight. The position of human recoverin is indicated by the *open arrow*; its labeling disappears in the preabsorbed blot, and it comigrates with recombinant bovine recoverin (data not shown). Autoradiography was used to detect p30 (*arrowhead*).

noblastoma cells malate dehydrogenase migrates at 39 kDa, making it an unsuitable candidate for p30 (Fig. 8). A two-dimensional immunoblot with antiserum raised against bovine recoverin showed that human recoverin has a similar molecular weight as p30 but a more acidic isoelectric point (Fig. 9).

To summarize, two-dimensional gel analysis of forskolin- and camptothecin-treated Y79 human retinoblastoma cells has revealed the existence of a protein, called p30, of $M_r \approx 30$ kDa and pI ≈ 5.7, which increases in radiolabeling intensity in parallel with increasing melatonin synthesis. Radiolabeling of p30 is already increased after the first hour of stimulation and remains elevated for at least 6 hr. The results of pulse–chase experiments suggest that forskolin and camptothecin stimulate the synthesis of p30, rather than prevent its degradation. Protein p30 appears to be cytoplasmic in location and different from protein 14-3-3, cytosolic malate dehydrogenase, and recoverin.

Discussion

In Y79 retinoblastoma cells forskolin stimulates an increase in melatonin production, NAT activity, and cAMP levels through a protein synthesis-dependent mechanism (Pierce et al., 1989, Janavs et al., 1991). The topoisomerase I inhibitor camptothecin also stimulates cAMP, NAT activity, and melatonin levels in Y79 cells (Janavs et al., 1994). Here we demonstrate that forskolin and camptothecin each stimulate the synthesis of the same Y79 retinoblastoma cell protein, p30 ($M_r \approx 30$ kDa, pI ≈ 5.7), in parallel with the degree to which they stimulate melatonin production. This suggests that p30 may be involved in the modulation of NAT activity and melatonin by forskolin and camptothecin.

The activation of tryptophan hydroxylase, the first enzyme in the pathway of indoleamine synthesis from tryptophan, requires the η isoform of 14-3-3 protein (Ichimura et al., 1987). Probing of two-dimensional immunoblots with monoclonal antibodies raised against two isoforms of protein 14-3-3, however, suggests that it is unlikely that p30 is a member of the 14-3-3 protein family. In experiments similar to ours, Voisin et al. (1990) have recently demonstrated that norepinephrine and cAMP analogs increase the radiolabeling of a rat pineal protein, AIP 37/6 ($M_r = 37$ kDa, pI = 6), which has been tentatively identified as cytoplasmic malate dehydrogenase (cMD, D. C. Klein, personal communication) and has characteristics similar to p30. A one-dimensional immunoblot comparing protein 14-3-3 and cMD in Y79 cells, however, shows that cMD migrates at 39 kDa, while p30 has a lower molecular weight than 14-3-3. An original candidate for rat pineal AIP 37/6 was HIOMT;

its molecular weight of 37–39 kDa in the monomeric form in several species (Nakane et al., 1983) also rules it out as a candidate for p30. Finally, a two-dimensional immunoblot probed with an antibody against recoverin, a 26 kDa calcium-binding protein found in the retina and the pineal gland of various vertebrate species (Dizhoor et al., 1991; Korf et al., 1992), shows that p30 fails to comigrate with the human isoform of recoverin.

A possible candidate for the identity of p30 is NAT. There are two forms of NAT, both cytosolic: arylamine-NAT and arylalkylamine-NAT (reviewed in Voisin et al., 1984). Arylamine-NAT does not display a circadian rhythm in its activity and is found primarily in the liver, but is also present in the kidney, in two forms in the pineal gland (Voisin et al., 1984), and in Y79 cells where it is stimulated by butyrate but not cAMP analogs (Gaudet et al., 1993). Human arylamine-NAT has been cloned and has a molecular weight of ~34 kDa (Ohsako and Deguchi, 1990). In contrast, arylalkylamine-NAT activity fluctuates in a circadian fashion and is present in vertebrate pineal glands and retinas; it is also found in Y79 retinoblastoma cells, where its activity is stimulated by cAMP analogs but not butyrate (Gaudet et al., 1993). Pineal arylalkylamine-NAT has been partially purified by activity and antibody assays and appears to migrate as a 30 kDa monomer on sizing columns in sodium citrate buffer (M. A. A. Namboodiri, personal communication). Unfortunately, the instability of arylalkylamine-NAT has so far precluded the successful cloning of the gene. The time course of p30 induction by forskolin is consistent with the stimulation of NAT activity by the same agent, which plateaus within the first 3 hr (Janavs et al., 1991). Thus, based on its cytosolic location, its molecular weight and its relationship to melatonin production, arylalkylamine-NAT is a reasonable candidate for the further investigation of the identity of Y79 protein p30. Positive identification of p30 awaits its purification and microsequencing.

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