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Expression and Production of Stathmin in Growth Plate Chondrocytes is Cell-Maturation Dependent

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Abstract Growth plate cartilage is comprised of linear columns of chondrocytes with the least differentiated cells at one end and the terminally differentiated cells at the other end. Rat costochondral chondrocytes can be divided into the resting cell zone (reserve cell zone), which contains relatively immature chondrocytes (RC cells), and the phenotypically more mature prehypertrophic and upper hypertrophic cell zones, which together may be termed the growth zone chondrocytes (GC cells). When grown separately in monolayer culture, they continue to express their zone-specific phenotype, providing a model for assessing cell-maturation–dependent expression of molecules associated with differentiation. Stathmin (also called prosolin, Op18, p19, 19K, and others) is a highly conserved, phosphorylated cytosolic protein with apparent ubiquitous expression. Although its exact function is unknown, stathmin is considered to be a messenger phosphorylated protein, it plays a role in tubulin stability, and it may participate in both general and specific regulatory pathways. One uniform observation is that the expression of stathmin protein decreases in all cells as they become more terminally differentiated in culture. There have been no published data regarding stathmin expression and production in chondrocytes. This study was based on the hypothesis that stathmin exists in chondrocytes and that the mRNA and protein levels decline in the GC cell with respect to the RC cell. Stathmin mRNA levels were determined and quantitated by reverse transcription-polymerase chain reaction (RT-PCR) and northern blots. Protein levels were determined using immunoblots. It was found that stathmin exists in chondrocytes and that RC cells express approximately twice the level of mRNA and protein to that found in GC cells. The results support the hypothesis and suggest that the level of stathmin expression and production in culture is related to the level of differentiation of RC and GC cells in vivo. J. Cell. Biochem. 79:150–163, 2000.

Key words: chondrocyte cultures; stathmin; cell maturation; growth plate

Stathmin is a phosphorylated cytosolic protein with expression occurring in a vast number of tissues and species [Chneiweiss et al., 1989; Koppel et al., 1990]. It is a member of a family of related proteins [Sobel et al., 1989; Ozon et al., 1997] and appears to be well conserved throughout the evolution of vertebrates [Rowlands et al., 1995]. Human and rat stathmin protein sequences vary by only one conservative amino acid substitution [Maucuer et al., 1990]. This high degree of conservation strongly suggests that stathmin serves an important function in mammalian cells [Okazaki et al., 1993b; Rowlands et al., 1995; Maucuer et al., 1993; Koppel et al., 1990].

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Stathmin exists as two isoforms, α and β, that differ by a post-translational modification but consist of the same 149 amino acids. In addition, stathmin contains four serine residues that may be phosphorylated via serine/threonine kinases [Beretta et al., 1993], resulting in at least 14 molecular forms [Beretta et al., 1989]. As a result of this heterogeneity, stathmin has been described under a variety of names [Braverman et al., 1986; Pasmantier et al., 1986; Friedrich et al., 1988; Hanash et al., 1988; Peyron et al., 1989; Ferrari et al., 1990; Schubart et al., 1992; Hoelscher and Ascoli, 1993;].

Stathmin is expressed in proliferating cells in culture [Rowlands et al., 1995]. It has been described as a messenger-relay phosphorylated protein that may participate in both general and specific regulatory pathways. Recent research has focused on its interaction with microtubules, and it has been found to play a role in tubulin stability [Belmont and Mitchison, 1996; Belmont et al., 1996; Marklund et al., 1996; Curmi et al., 1997; Di Paolo et al., 1997; Horwitz et al., 1997; Jourdain et al., 1997; Larsson et al., 1997; Tournebize et al., 1997; Gradin et al., 1998; Lawler, 1998]. The results of these studies indicate that stathmin destabilizes tubulin by interacting with heterodimers and that increased phosphorylation of stathmin removes this interaction. Nondividing cells rely on microtubules for maintenance and function of the cytoskeleton. However, during mitosis, stathmin is involved in spindle formation and cytokinesis, suggesting that it is a key protein in regulating postproliferative differentiation [Balogh et al., 1996; Belmont et al., 1996; Schubart et al., 1996; Andersen et al., 1997; Laird and Shalloway, 1997; Lawler et al., 1998; Pedrozo et al., 1998; Andersen, 1999].

Although the role and expression of stathmin may vary for each specific cell type, one uniform observation is that the expression of stathmin protein decreases in all cells as they become more differentiated in culture [Schubart et al., 1992]. Both the level of production and the degree of phosphorylation of stathmin have been associated with changes in cell proliferation [Cooper et al., 1989; Melhem et al., 1991; Koppel et al., 1993; Okazaki et al., 1993a], differentiation [Doye et al., 1992a; Gratiot-Deans et al., 1992; Schubart et al., 1992], and development [Grinell, 1978; Koppel et al., 1990;]. This may be important in maturation of mesenchymal cells, like osteoblasts and chondrocytes, because the onset of differentiation in culture is associated with decreased proliferation [Stein et al., 1990; Stein and Lian, 1993]. Stathmin mRNA and proteins have been found in human and rat osteoblast-like cells, including SAOS-2 human osteosarcoma cells, ROS 17/2.8 and ROS 25/1 rat osteoblast-like osteosarcoma cells, normal and transformed human osteoblast-like cells, and fetal rat osteoblast-like cells [Schubart et al., 1992; Kumar and Haugen, 1994].

The studies described above have been performed in vitro using cells at various, but unknown, states of maturation in their respective lineage cascades. Although the results tell us about the behavior of stathmin in culture, they cannot be correlated with the state of maturation of the cell in its lineage in vivo. We have developed a cell culture model that enables us to compare chondrocytes at two well-defined and distinct maturation states based on their zone of origin in the costochondral growth plate cartilage of rats [Boyan et al., 1994a]. Chondrocytes in the growth plate proceed through a regulated progression of events. Because the cells are aligned in columns, the can be divided into zones based on distinct histologic and biochemical characteristics [Iannotti, 1990]. The resting zone (reserve cell zone) contains chondrocytes that have not yet been committed to terminal differentiation with respect to calcification. These cells maintain a proteoglycan-rich extracellular matrix that does not support mineral formation. A zone consisting of proliferating chondrocytes follows the resting zone. Postproliferative cells undergo terminal differentiation. After a period of maturation (prehypertrophic cell zone), these cells become hypertrophic (hypertrophic cell zone) and ultimately calcify their matrix. The resting zone and the prehypertrophic/upper hypertrophic cell zones (growth zone) can be cleanly dissected from the other zones and adjacent bone. Confluent cultures of cells isolated from resting zone (RC cells) and growth zone (GC cells) costochondral cartilage continue to express their zone-specific phenotype, even after four passages in monolayer culture [Boyan et al., 1988b; Schwartz et al., 1988a; Schwartz et al., 1988b; Boyan et al., 1992].

We have used this model to assess the role of cell-maturation state in chondrocyte response

to a variety of regulatory factors. In the present study, we examined whether stathmin mRNA and protein are present in chondrocytes, and if so, whether the levels are correlated with the maturation state of the cell in endochondral development. Our hypothesis was that in culture both mRNA and protein levels would be lower in GC cells, which in vivo are postproliferative and at a more mature phenotypic state in the endochondral lineage than are RC cells.

MATERIALS AND METHODS

Reagents are from Sigma (St. Louis, MO) unless otherwise noted.

Cell Culture

Ribs were removed by sharp dissection from Sprague-Dawley rats (Charles River Breeding Labs, Wilmonton, MA) weighing 120 ± 20 g and placed in Dulbecco's modified Eagle's medium (DMEM) until the costochondral cartilage could be removed. The costochondral resting zone and adjacent growth zone cartilage were carefully separated, discarding the intervening proliferating cell zone and the calcified cartilage. This technique takes advantage of the linear architecture of the growth plate and limits contamination with cells at other stages of differentiation/maturation. The tissue was then washed two times for 20 min each in Hank's balanced salt solution (HBSS; Gibco BRL, Rockville, MD) containing 1% penicillin-streptomycin. Cartilage was then sequentially digested with 1% trypsin (Gibco BRL) for 1 h, and 0.02% collagenase (type II, Worthington, Freehold, NJ) for 3 h. Both enzymes were dissolved in HBSS. Cells were separated from tissue debris by filtration through a 40-μm nylon cell strainer (Falcon, Franklin Lakes, NJ), followed by centrifugation of the filtrate at 500*g* for 5 min. Cell viability was 95%, as demonstrated by Trypan blue exclusion. Cells were plated in T-75 flasks at 10,000 cells/cm^2 for resting zone cells and 25,000 cells/cm^2 for growth zone cells. Incubation was conducted in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin and 50 μg/ml sodium ascorbate in an atmosphere of 5% CO_2 at 37°C and 100% humidity for 24 h. At the end of this time, culture media were replaced three times per week. At confluence, cells were subcultured to either T-75 or T-150 flasks using the same plating densities as before and allowed to return to confluence. Both cell populations take five to eight days (seven-day average) to reach confluence in primary culture and in each subculture. Fourth-passage cultures were used for these experiments because a number of studies have demonstrated that these cells retain their differential phenotype up to this many passages in culture [Boyan et al., 1988a; Boyan et al., 1988b; Schwartz and Boyan, 1988; Schwartz et al., 1988a; Schwartz et al., 1988b; Boyan et al., 1989; Schwartz et al., 1989; Schwartz et al., 1990; Schwartz et al., 1991; Schwartz et al., 1992a; Schwartz et al., 1992b; Schwartz et al., 1993; Boyan et al., 1994b; Dean et al., 1994; Schwartz et al., 1995].

ROS 17/2.8 rat osteoblast-like osteosarcoma cells were plated at 10,000 cells per T-75 flask and grown under the same conditions, harvesting at confluence, which takes two to three days.

Stathmin mRNA Levels

To determine whether stathmin expression varied with chondrocyte maturation state, we examined RC and GC cells for stathmin mRNA by reverse transcription-polymerase chain reaction (RT-PCR) and Northern analysis.

Total RNA was extracted from fourth-passage cultures using a guanidinium thiocyanate procedure [Chirgwin et al., 1979] or TRIzol reagent (Gibco BRL) [Chomczynski and Sacchi, 1987]. The isolated RNA pellet was resuspended in 40 μl of diethyl pyrocarbonate (DEPC) treated water (0.2 ml diethyl pyrocarbonate [Fluka Chemical Corp., Milwaukee, WI] in 100 ml double distilled H_20 [ddH_20] and autoclaved) [Gilman, 1999]. A 4 μl aliquot was then diluted 1:250 in DEPC water. The total RNA was calculated by quantitation from the spectrophotometic reading (Beckman, Fullerton, CA) where $C_{\mu g/ml} = A_{260nm}/0.025$ [Gallagher, 1999].

Optimal primers were constructed from the rat stathmin cDNA sequence (GenBank/EMBL Data Bank accession number J04979) [Doye et al., 1989] using OLIGO software (National Biosciences, Plymouth, MN). Primers for stathmin composed of 25 nt were specifically designed to cross the fourth intron, thereby adding confidence that the product is a result of mRNA and not DNA contamination. Oligonucleotide primers for stathmin and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were synthesized commercially (Genosys Biotechnologies,

Inc., The Woodlands, TX). Primer sequences for the stathmin and GAPDH are as follows. Stathmin, the upstream primer: 5′-AAA-ATGGAGGCTAACAAAGAGAAAC-3′ (sense, 478–502, located in exon 4); the downstream primer: 5′-AGGGAGGAAAAAGTAAATGACA-CTG-3′ (antisense, 680–704, located in exon 5). GAPDH, which was characterized previously [Schmitz et al., 1995; Schmitz et al., 1996], the upstream primer: 5′-TGATGGGTGTGAACC-ACGAGAAATA-3′ (sense, 779–803); the down-stream primer: 5′-AACGGATACAATGGGG-GTAGGAACA-3′ (antisense, 779–803). Oligo-nucleotide primers were selected based on the ability to repeatably produce the predicted PCR products. Optimization of the PCR reaction for each primer set and cell-specific RNA was accomplished for determination of enzyme concentration, dNTP solutions, magnesium concentration, annealing and primer extension temperatures, and cycle number necessary to reach a linear amplification as well as plateau [Innis and Gelfand, 1990], which resulted in the use of stock solutions called A-Mix and T-Mix [Chen et al., 1993]. Each 6.25 μl of A-Mix contained 1.6 μl 10× Cetus PCR buffer (Perkin Elmer, Wellesley, MA), 0.84 μl 1M Tris (pH 8.3; Gibco BRL), 1 μl bovine serum albu-min (BSA; 5 mg/ml), 0.5 μl dithiothritol (0.5 M), 1.36 μl $MgCl_2$ (0.01 M), 0.4 μl dNTP (25 mM; Pharmacia Biotech, Picscataway, NJ) 0.5 μl RNasin (40 Units/μl; Fisher, Houston, TX), 0.05 AMV (20 Units/μl; Gibco BRL). Each 78 μl of T-Mix contained 26.7 μl DNase-free H_2O, 20 μl 300 mM Tris pH 9.5, 8 μl Taq buffer (Promega, Madison, WI), 6.0 μl DMSO, 8.0 μl $MgCl_2$, 0.8 μl dNTP (25 mM; Pharmacia), and 0.5 μl Taq (5 Units/μl; Fisher).

RT-PCR was performed using oligonucleo-tide primers. The sense primers were diluted in DEPC water to 100 ng/μl and the antisense diluted to 200 ng/μl. Labeling of the 5′ primer for quantitative PCR was accomplished for each reaction tube at 1 μCi activity per reac-tion and used 10 μl at 100 ng/μl as follows: 1 μl (1 μg) of sense primer (1μg/μl), 1 μl (150 μCi; adjusted for specific activity) of [α-^{32}P]ATP (150 mCi/ml; specific activity 1.0; NEN Life Science Products, Boston, MA), 1 μl of T4 ki-nase 10× buffer (Fisher), 0.5 μl of T4 kinase (1 U/10 pmol = 15 U/1 μg; Fisher), qs to 10 μl with DEPC H_2O: total of 10 μl of 100 ng/μl sense primer (15 μCi/μl). This was heated at 37°C for 20 min, then the kinase was inacti-vated by heating at 90°C for 2 min. The RT reaction used 1 μl of antisense primer and 1 μg of total RNA diluted in DEPC H_2O qs to 13.75 μl and then heated to 68°C for 10 min followed by 5 min on ice. The tube then had 6.25 μl of A-Mix added and the RT reaction was run for 40 min at 42°C followed by 5 min on ice. The standard PCR reaction then followed by adding 2 μl of sense primer and 78 μl of T-Mix then run at the following times and tempera-tures: 94°C for 1.5 min one time, then cycled 28 times at 56°C for 1 min, 72°C for 1 min, and 94°C for 20 s, ending at 4°C on a PTC-100, programmable thermal controller, Model 60, (MJ Research, Inc., Watertown, MA). Initially, aliquots were drawn every two cycles from 16 to 32 cycles to determine detectable RT-PCR product and where linear amplification oc-curred [Innis and Gelfand, 1990]. Relative dif-ferences in mRNA levels were determined by comparing aliquots from the RT-PCR reaction tube at 26 cycles of RC cells and 28 cycles for GC cells (within the linear amplification of the reaction). Along with the aliquots, BioMarker low molecular weight markers and BioTracker 6× gel loading buffer (BioVentures, Inc, Mur-freesboro, TN) were used as recommended for nucleotide markers for verification and visual-ization of the PCR reaction and product. The aliquots of 15 μl were added to 3 μl 8× sample buffer (3.125 mg/ml bromophenol blue, 1.25 g/ml Ficoll type 400 and 250 μl glycerol qs to 1 ml with sterile ddH_2O) and heated to 65°C for 5 min. The sample was then loaded imme-diately on 5% polyacrylanide gels using TBE buffer (0.002 M EDTA, 0.09 M boric acid, and 0.09 M Tris, pH 8.0) on an SE 250 Mighty Small II apparatus (Hoefer Pharmacia Biotech, Inc., San Francisco, CA), and run at 30–35 milliamps per gel for 20–25 min. The gels were then stained with 10 μg/ml ethidium bromide in TBE buffer for 30 s, destained in TBE buffer for 1 min, visualized by UV and photographed with a Polaroid CR-9 Land camera using 667 film (Polaroid, Cambridge, MA). The ^{32}P gels were dried, wrapped, and placed in a Phosphor-Imaging screen and analyzed using Image-Quant software (Molecular Dynamics, Sunny-vale, CA). Previously accepted and published techniques for the relative production of mRNA as compared to a housekeeping enzyme, GAPDH, have been performed [Chen et al., 1992; Bouaboula et al., 1992] and confirmed in our lab [Schmitz et al., 1995; Schmitz et al.,

1996]. These techniques have provided the protocols for these experiments in determining the basal levels of stathmin mRNA by RT-PCR techniques. PCR products for GAPDH have been sequenced and verified through the GenBank/EMBL Data Bank sequence information (unpublished data). The levels of stathmin mRNA were quantitated according to the method of Chen et al. [1992] using GAPDH as a denominator [Fort et al., 1985; Chen and Klebe, 1993; Schmitz et al., 1995; Schmitz et al., 1996].

The stathmin PCR products were sequenced to confirm mRNA specificity using the T7 Sequenase PCR sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) as directed using [α-^{32}P]ATP. This technique only allows for the sequencing from the PCR product, as all other nucleotides were digested by the exonuclease and the free nucleotides were inactivated. Compressions necessitated the addition of formamide and the use of glycerol-tolerant buffer in concentrations recommended in the directions. The PCR reaction was run to 32 cycles, as described above, and the total product quantitated as above for cDNA where $C_{\mu g/ml} = A_{260\ nm}/0.027$, which equals about 5 ng/μl of product. The sequencing reaction used 7.5 μl of product, 1.5 μl shrimp alkaline phosphatase, and 1.5 μl exonuclease. Standard mix (5 μl) and 1 μl of 40-primer mix (1 μl primer and 4 μl ddH_2O) were used as a control for the reaction. Reactions were run in both the sense and antisense direction to overlap sequence due to the short product length. Six percent polyacrylamide-urea gels (21 $\times$ 40 cm) were made from Instagel stock and initiators. Instagel was made of 150 ml 40% stock acrylamide solution [380 g acrylamide and 20 g bisacrylamide qs to 1 liter deionized with 20 g mixed bed resin AG501-X8 (BioRad, Hercules, CA) for 2 h and filtered through 45 μm Nalgene filter (Nalge Company, Rochester, NY)], 100 ml 10$\times$ TBE and 460 g urea, qs to 1 liter filtered and degassed for 20 min, then stored in dark conditions at 4°C. A sealing gel was used first where 10 ml of the Instagel stock solution, plus 50 μl fresh 25% ammonium persulfate (APS) and 50 μl N,N,N′,N′tetramethylethylenediamine (TEMED) was added to initiate polymerization. The gel was poured from 40-ml Instagel solution plus 80 μl each of APS and TEMED, and allowed to set for 3 h. Gels were preheated at 1,400 volts constant power (35 watts and 25 mamps) for 20 min to stabilize at 55°C. Sequencing samples were heated to 95°C for 3 min and quenched in a −70°C frozen isopropyl alcohol block, then transferred to ice. Samples were loaded and run under the above conditions but at 50°C until the dye front ran off the gel, or as indicated by sequence results. The gels were removed and fixed in 10% methanol and 10% acetic acid for 30–45 minutes, transferred, dried for 1 h at 80°C, and Biomax MR film (Kodak, Rochester, NY) was exposed for 12–15 hours. The sequences were read manually, recorded, and compared to the known sequence.

Total RNA was extracted and quantitated as described above for RT-PCR. Northern analysis was performed with the NorthernMax system and Strip-EZ RNA kit (Ambion, Austin, TX). Samples of 17 μg total RNA in sample buffer (Ambion) per well or Millennium markers (Ambion) were run on 1% denaturing agarose (prepared with 10$\times$ denaturing gel buffer–formaldehyde/MOPS) for 2 h at 100 volts using an EasyCast B1 apparatus (Owl Separation Systems, Portsmouth, NH). RNA was transferred to a positively charged nylon membrane (Ambion) with the Turboblotter System (Schleicher and Schuell, Keene, NH) using the NorthernMax transfer buffer for 2 h. The membrane was then heated to 80°C for 15 min. RT-PCR products were amplified with modified antisense primers that have the T7 promoter sequence, 5′-TAATACGACTCACTATAGGGAGG-3′, attached to the 5′ end of the antisense primers (described above). Probes for stathmin cRNA or GAPDH cRNA probes were then synthesized with the Strip-EZ T7 Kit (Ambion) from RT-PCR products. The T7 transcription reaction (Ambion) is used to produce a labeled [α-^{32}P]UTP (NEN Life Science Products) stathmin cRNA (first hybridization) or GAPDH (second hybridization) cRNA probe. The reaction mixture was treated with DNase for 15 min at 37°C. The cRNA probes were separated from the unincorporated nucleotides with a Micro Bio-Spin P-30 column (BioRad, Hercules, CA). A scintillation counter was used to count 1 μl of the probe and radioactive counts were normalized for 5×10^7 dpm for hybridization. The membranes were hybridized for 12–18 h at 65°C using 10 ml Northern Max prehybridization/hybridization buffer (Ambion) with stathmin strippable [α-^{32}P]UTP-labeled cRNA probes using the Northern Max Kit protocols (Ambion). This was

followed by rinses using Northern Max wash buffer 1 (Ambion) using two low stringency washes for 5 min each at room temperature and two high stringency washes with wash buffer 2 for 15 min each at 65°C. The Northern blots were analyzed with a PhosphorImager screen and adjunctive ImageQuant software (Molecular Dynamics). The membranes were then striped using the Strip-EZ system and reprobed with the GAPDH cRNA probe, as described above. Northern analysis protocols have been previously described for stathmin where the expected size of the transcript should be observed at about 1.1 kb [Koppel et al., 1990; Amat et al., 1991; Doye et al., 1992a; Ghosh et al., 1993; Schubart et al., 1992]. The expected size of the GAPDH transcript should be observed at about 1.5 kb. Differences in the arbitrary counts for target RNA (stathmin) versus control RNA (GAPDH) were used to determine alteration of expression.

Stathmin Protein Levels

To determine if stathmin protein in the cells reflected differences in mRNA levels, stathmin was purified from cultures of RC and GC cells as described above.

Cytosolic proteins were harvested from confluent fourth-passage costochondral chondrocyte cultures grown as described above. A modification of the rapid scheme for purification of stathmin was applied that provides concentrated protein fractions suitable for polyacrylamide gel electrophoresis (PAGE) analysis [Beretta et al., 1989]. T-150 flasks of confluent chondrocyte cultures were harvested by scraping in 3.5 ml of ice-cold homogenization buffer (10 mM TrisHCl pH 7.4, 0.02% NaN_3, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 15 mM $Na_4P_2O_7$, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin). This was then transferred to and homogenized in a Tenbroeck tissue grinder on ice. The lysate was transferred to a 3.5-ml polyallomer tube and centrifuged in a Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) using a SW 50.1 rotor at 100,000*g* for 10 min. The pellet was discarded and the supernatant retained, transferred to 20 × 150 mm Pyrex test tubes (Corning, Inc., Corning, NY), incubated in a boiling water bath for 10 min, then cooled on ice. The samples were then transferred to a clean polyallomer tube and centrifuged again at 100,000*g* for 10 min. The pellet was discarded, the supernatant was retained and defined the "stathmin-enriched fraction" (SEF). The samples were then concentrated for SDS-PAGE analysis by transferring to 6,000–8,000 MWCO Spectra/Por 1 dialysis tubing (Spectrum Laboratories, Inc., Gardena, CA) for dialysis against buffer containing 2 mM Tris-HCl, 1 mM NaCl and 0.008% NaN_3. The samples were then lyophilized and stored at 4°C until reconstituted in 50 μl of phosphate buffered saline (PBS). Boiling does not affect either the phosphorylated or nonphosphorylated forms of stathmin [Sobel et al., 1989].

Protein concentrations were determined by a modified miniaturization of the method of Lowry et al. [1951] using the BCA protein reagent kit #23225 (Pierce, Rockford, IL) with BSA used as a standard. Samples were loaded using equal amounts of protein. Molecular weight markers (MWM) used were either ECL Western blotting MWM (Amersham Pharmacia Biotech), MultiMark Pre-stained, or See-Blue MWM (Novex, San Diego, CA). Recombinant stathmin (RST; CalBiochem, San Diego, CA) standards, MWMs, or samples for RC and GC cells using either 10, 15, or 20 μg of the SEF were diluted in 2× sample buffer (0.125 M Tris-Cl pH 6.8, 4.1% SDS, 10% glycerol, 0.02% bromophenol blue, 2% 2-mercaptoethanol). Empty lanes received 2× buffer only. These samples were then heated to 100°C for 5 min and electrophoresed on precast 15% polyacrylamide mini gels (BioRad, Hercules, CA) according to the procedure of Laemmli [1970], at 100 V for 1.6 h on a Ready gel cell (BioRad).

Immunoblotting

A polyclonal antiserum against stathmin was generated in rabbits using a synthetic peptide based on a slight modification of the peptide I sequence NH2-CASGQAFELILSPN-COOH (amino acids 15–27) as the antigen [Koppel et al., 1990]. Previous studies demonstrated that this peptide could be used to generate antisera with high specificity for stathmin [Koppel et al., 1990]. The peptide and antiserum were produced by Alpha Diagnostic International (San Antonio, TX). Sodium azide (0.1%) was added as a preservative and the antiserum was stored at 4°C. Both fetal rat brain (FRB) and adult rat brain (RB) were included as controls. Stathmin is abundant, well characterized [Beretta et al., 1989; Chneiweiss et al., 1989; Koppel et al., 1990; Amat et al., 1991; Doye et al., 1992b; Beretta et al.,

1993; Ozon et al., 1997; Zugaro et al., 1998] and the FRB contains more than the RB. Therefore, initial controls consisted of FRB and RB homogenates enriched for stathmin (as described above, using 10 volumes of buffer per mg tissue), and recombinant human stathmin (RST; Calbiochem) diluted 10 μg/μl in PBS and frozen in 10-μl aliquots at −70°C. The day of the experiment an RST aliquot was then thawed and rediluted with PBS to 1 ng/μl, and used at varied ng concentrations for standards on the immunoblot. Peptide blocking of the antibody, as described below, was also performed to demonstrate specificity.

PAGE gels were equilibrated in Towbin transfer buffer (25mM Tris, 192mM glycine, 15% methanol, 0.05% SDS, pH 8.3) for 10 min. Transfer to a 0.2-μm Immun-Blot PVDF membrane (BioRad) with the transfer buffer in a Mini Trans-Blot electrophoretic transfer cell wet transfer apparatus (BioRad) was accomplished in 1.2 h at 100 V. Transfer was confirmed by post-transfer silver staining of the gel (Silver Stain Plus; BioRad). All incubations or washes were performed on an orbital shaker at low-medium speed. Following transfer, the membranes were equilibrated in 0.05% Tween 20-PBS (T-PBS) for 15 min. Membranes were blocked for nonspecific interaction in membrane blocking solution (ZYMED Laboratories, Inc., South San Francisco, CA) for either 1 h at room temperature or overnight at 4°C. The membranes were then washed a minimum of three times for 15 min each in T-PBS. Antibodies were diluted in the membrane blocking solution at 1:3,000 (vol/vol) for the anti-stathmin sera and anti-rabbit ECL-HRP (Amersham Pharmacia Biotech), and 1:500 for the streptavidin-horseradish peroxidase conjugate (SHP; Amersham Pharmacia Biotech). To perform peptide blocking of the antibody, an additional antibody-blocking solution was made: 10 μg/ml of the peptide used to produce the antibody sera was added to the 1:3,000 (vol/vol) antibody dilution and incubated for 1 h at room temperature. For the antibody-blocking experiment, the membrane was cut to separate the individual lanes and alternately incubated with the antibody solution with or without the antibody-blocking peptide. For quantitation experiments, the SeeBlue markers were index-marked with a pencil to prevent loss during incubations and washes, and the ECL markers were cut away from the other lanes. Following transfer, equilibration, and blocking, the membranes were washed a minimum of three times for 15 min each in T-PBS. They were then incubated with the diluted anti-stathmin solution for either 1 h at temperature or overnight at 4°C. Membranes were washed a minimum of three times for 15 min each in T-PBS. Contemporaneously, ECL membranes were incubated in the SHP solution and the membranes with samples were incubated with the secondary diluted anti-rabbit antibody solution for 1 h at room temperature. All membranes were washed a minimum of three times for 15 min each in T-PBS. The membranes were then incubated for 1 min with the ECL Western blotting reagents (Amersham Pharmacia Biotech), blotted to remove excess liquid, placed in a protective plastic sleeve, placed in a film cassette with ECL Hyperfilm (Amersham Pharmacia Biotech), and developed in an automatic film processor.

Quantitation of the stathmin protein in RC and GC cells was then accomplished using the RST to establish a standard curve. New RST was used from −80°C aliquots for quantitation. It is imperative that fresh RST is used to make standard curves. It was found that the recombinant protein was subject to degradation over a period of one to two weeks when stored at 4°C in PBS without preservatives, making direct quantitation inaccurate. The RST was loaded on 15% SDS-PAGE gels at varied ng amounts as well as SEF samples from RC or GC cells. Immunoblots of the gels were performed and the resulting films analyzed using NIH Image 1.61. A standard curve was derived from the RST standards used to calculate the unknown amount of stathmin in the samples.

To determine if stathmin was phosphorylated, immunoblots were also probed with rabbit polyclonal anti-phosphoserine antiserum (ZYMED Laboratories, Inc.). Antibodies were diluted 1:500 (vol/vol) in membrane-blocking solution as described in the manufacturer's instructions. Stathmin isolated from RC cells was compared to FRB and RST. Immunoblots were initially probed with anti-phosphoserine antibody, stripped and reprobed with anti-stathmin peptide antibody. The resulting films were overlaid and compared.

Analysis and Statistics

Films from immunoblotting ECL experiments were scanned in transparency mode to

a TIF file and analyzed by NIH Image 1.61 software (NIH, Bethesda, MD) on a Power Macintosh 7200/120 (Apple, Cupertino, CA). Quantitative data from NIH Image and PhosphorImager data from ImageQuant software were analyzed with StatView 5.0.1 software (SAS Institute Inc., Cary, NC). The data presented are from one of two or more comparable independent experiments with an n = 3–6 samples. Significance between groups was determined by Bonferroni's post-hoc analysis using $P < 0.05$. Results are graphed using Deltagraph 4.05 software (SPSS Inc., Chicago, IL).

RESULTS

Expression of Stathmin Genes

Using the stathmin primers under the optimized conditions determined in this study, both RC and GC cells produce single band RT-PCR products at the expected size of 227 nt (Fig. 1). This band was also found in mRNA from ROS 17/2.8 cells, which have been shown previously to have stathmin [Kumar and Haugen, 1994]. Because the amplified product was very short, it was sequenced in both directions and overlapped. By comparison with published sequence for rat stathmin (GenBank/EMBL Data Bank accession number J04979) [Doye et al., 1989], the results confirmed the product in RC and GC chondrocytes, as well as in ROS 17/2.8 cells, to be stathmin. A significant difference in relative levels between RC cells and GC cells were evident when bands were normalized to background and GAPDH levels (Fig. 2). Northern blot analysis confirmed expression of stathmin mRNA in RC and GC cells (Fig. 3). Probes hybridized to the region of 1.1 kb for stathmin and 1.5 kb for GAPDH. A significant difference in relative levels between RC and GC cells were evident when the bands were normalized to background and GAPDH levels. RC cells had 1.8 times as much stathmin mRNA as was present in GC cells.

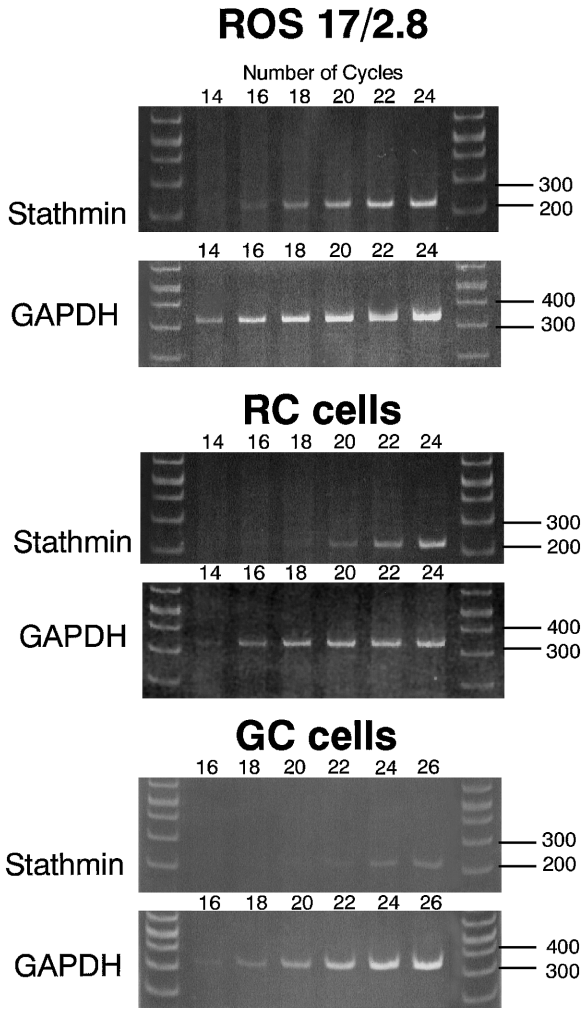

Fig. 1. Basal levels of stathmin mRNA in ROS 17/2.8 cells and confluent, fourth-passage cultures of resting zone (RC) and growth zone (GC) chondrocytes. Total RNA was extracted and stathmin expression examined by reverse transcription-polymerase chain reaction. The expected 227 nt stathmin and 332 nt glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA fragments were amplified from the total RNA and analyzed by 5% polyacrylamide gel electrophoresis and ethidium bromide staining. GC stathmin was expressed at lower levels, requiring increased cycles for detection.

Production of Stathmin Protein

Stathmin protein was also present in the RC and GC cells. Extracts of both RC and GC cells exhibited a single immunoreactive band at 19 kDa, which comigrated with the immunopositive recombinant stathmin used as the positive control (Fig. 4). Similarly, immunopositive bands for stathmin appeared at the expected size of 19 kDa for extracts of fetal rat brain and adult rat brain. Specificity for stathmin was demonstrated by the lack of immunoreactivity on Western blots where the peptide used to generate the sera was used as a blocking agent (Fig. 4).

When the amount of immunoreactive protein was calculated based on an RST standard curve performed on each gel (Fig. 5), RC cells contained more stathmin protein than the GC

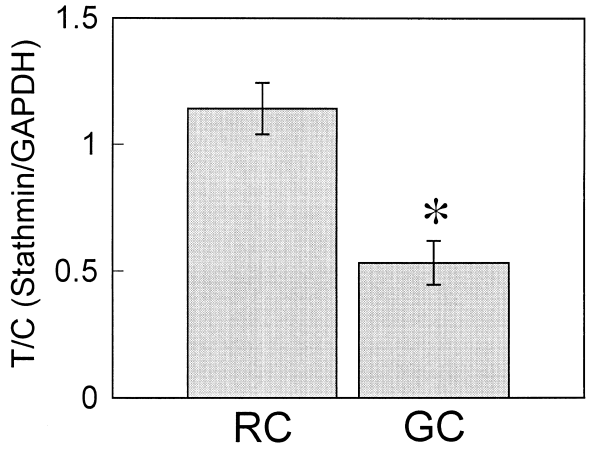

Fig. 2. Basal stathmin mRNA expression derived from reverse transcription-polymerase chain reaction (RT-PCR) products for resting zone (RC) and growth zone (GC) cells run on 5% polyacrylamide gels and quantitated by PhosphorImaging of incorporated [α-^{32}P]ATP labeled RT-PCR sense primers. Expression of the stathmin gene was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Target (T) values were corrected using GAPDH as a control (C) and are the T/C mean ± SEM of mRNA from six independent cultures. Data presented are from one of two separate experiments, both with comparable results. $^*P < 0.05$.

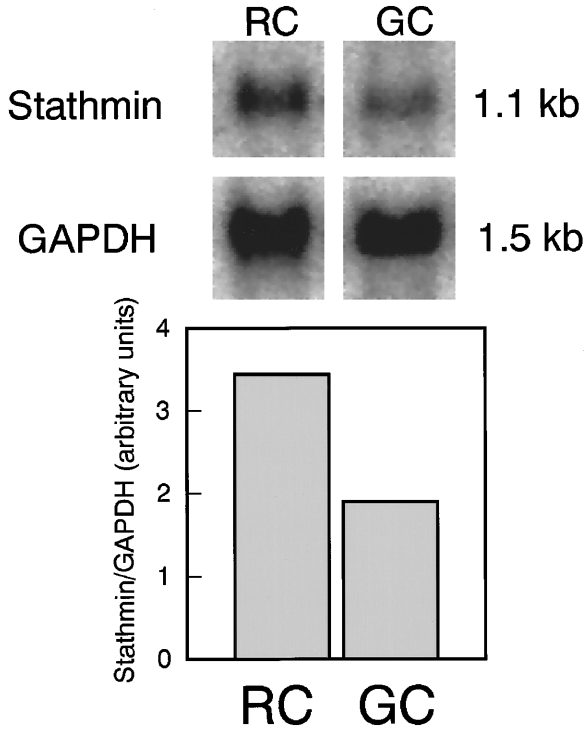

Fig. 3. Northern blot analysis of stathmin expression in resting zone chondrocytes (RC) and growth zone chondrocytes (GC). Seventeen μg total RNA extracted from confluent fourth-passage RC and GC cells was run per lane, transferred to an Ambion BrightStar positively-charged membrane and hybridized with [α-^{32}P]UTP-labeled cRNA probes. Stathmin mRNA was quantitated by PhosphorImaging of hybridized probes. Expression of the stathmin gene was normalized to the housekeeping gene GAPDH.

cells (Fig. 6). RC cells were found to contain 0.970 ± 0.043 ng/μg SEF, and GC cells contained 0.367 ± 0.038 ng/μg SEF.

Neither RC nor GC cells exhibited evidence of phosphorylated stathmin in the confluent cultures as determined by anti-phosphoserine antibody experiments that included phosphatase inhibitors during harvest (data not shown). The anti-phosphoserine immunoblot displayed very strong immunopositive bands in the RC lanes at about 32 and 35 KDa, where a strong doublet appeared. A single, very faint immunopositive band appeared in the same region of 35 KDa for the FRB sample. However, no immunopositive bands appeared in the region of stathmin (18–22 KDa) in any of the samples. Stathmin was present in these samples because the anti-stathmin antibody revealed immunopositive bands in all lanes after stripping the membrane and reprobing. None of the stathmin bands comigrated with the phosphoserine bands. (Results not shown).

DISCUSSION

The results of this study demonstrate for the first time that confluent cultures of rat costochondral growth plate chondrocytes express mRNA for stathmin and produce stathmin protein. Moreover, the amount of stathmin mRNA and protein correlate with the endochondral maturation state of the cells in vivo. RC cells, derived from the less mature resting zone of the growth plate, contain about two times as much mRNA and about 2.5 times as much protein as GC cells, derived from the more mature growth zone. Under constitutive culture conditions, stathmin does not appear to be phosphorylated in either cell type, suggesting that phosphorylation of the protein occurs as a consequence of regulation, which was not examined in this study. To obtain a comparable amount of RC and GC cells at confluence, RC cells are seeded at a lower density than GC cells, reflecting their cell density in vivo. RC cells are more dispersed in their extracellular matrix whereas GC cells are more densely arranged in linear columns. Neither population of cells is proliferative in vivo, but when cultured in vitro, both populations are capable of

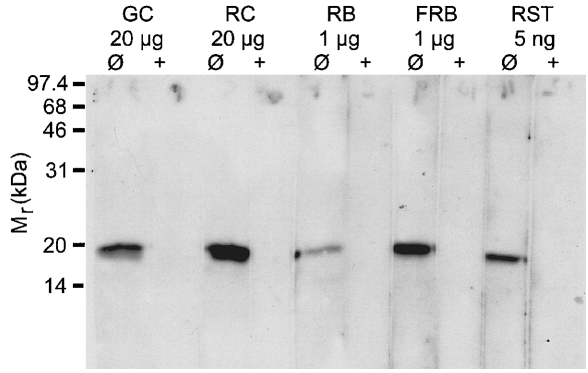

Fig. 4. Immunoblot of stathmin using the stathmin peptide (15–27) to block the anti-stathmin antibody. Stathmin-enriched protein, from resting zone (RC) and growth zone (GC) chondrocytes (20 µg), rat brain homogenate (RB; 1 µg), fetal rat brain homogenate (FRB; 1 µg), and recombinant stathmin (RST; 5 ng), was separated cells and transferred to a membrane. Migration on the gels was compared to RST. Duplicate lanes of each sample were run and the membrane cut into strips to separate all the lanes. Strips were either incubated with anti-stathmin antibody solution (∅) or with antibody solution pretreated with stathmin peptide (+). Stathmin is expected to run from 19 kDa (unphosphorylated) to 23 kDa (all sites phosphorylated).

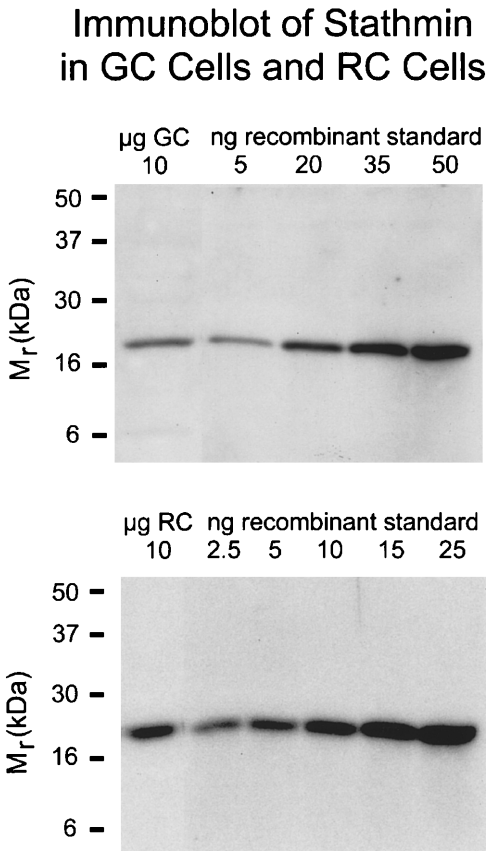

Fig. 5. Representative immunoblot analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of stathmin-enriched protein from growth zone (GC) cells (upper) and resting zone (RC) cells (lower). Recombinant stathmin (RST) was used to generate a standard curve. A regression curve was then derived from the standards and used to quantitate the test samples.

proliferation, with the rate of proliferation being greater in the RC cell cultures. This difference in proliferation may account for some of the difference in stathmin. However, we used confluent cultures for these experiments and at confluence, the number of chondrocytes in each type of culture is comparable. Proliferation still occurs in postconfluent cultures, but the amount of proliferation is also comparable in both cell types [Schwartz et al., 1989]. Moreover, the confluent cultures express their in vivo phenotype in a variety of ways, including cell-specific phenotypic characteristics [Boyan et al., 1988b], differential responsiveness to vitamin D metabolites [Boyan et al., 1994a], hormones [Schwartz et al., 1997], growth factors [Erickson et al., 1997; Schwartz et al., 1998b], and prostanoids [Schwartz et al., 1998a]. Thus, it is likely that the differences in stathmin observed in the present study reflect levels of stathmin in these cells in vivo.

The role that stathmin plays in differentiation is not completely understood, although it is clear that stathmin is related to the state of differentiation of the cell [Chneiweiss et al., 1989; Amat et al., 1990; Koppel et al., 1990; Sobel, 1991; Schubart et al., 1992; Rowlands et al., 1995]. Because stathmin interacts with tubulin and may alter cytokinesis, its levels may be an indication of a cell's ability to proliferate. Our observations support this hypothesis. RC cells, which are preproliferative in vivo, exhibit higher basal levels of stathmin than do GC cells, which are postproliferative in vivo. Previous studies examining the relationship of stathmin expression to proliferation and differentiation have used cytokines, hormones, or other agents to induce cell differentiation. The results presented here indicate that the amount of stathmin may be an intrinsic property of cells at specific maturation states, since the chondrocyte cultures were examined at confluence in the absence of exogenous factors to promote either proliferation or differentiation.

Increased amounts of stathmin are present in actively proliferating cells and reduced in terminally differentiated cells [Chneiweiss et

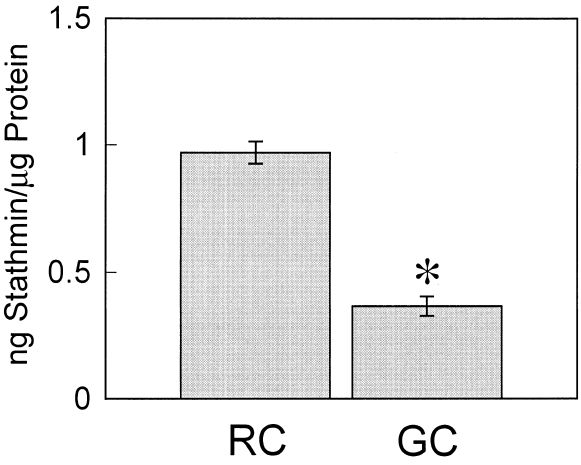

Fig. 6. Basal levels of stathmin protein production in confluent, fourth-passage cultures of resting zone (RC) and growth zone (GC) chondrocytes. RC and GC cell stathmin-enriched fraction (SEF) samples were normalized by loading equal amounts of protein (10 μg) in each lane. Densitometric analysis was performed using NIH Image 1.61 software. A regression curve was derived from the recombinant stathmin standards (RST) and used to quantitate stathmin in the RC and GC samples, with an average correlation coefficient of 0.98 for RCs and 0.99 for GCs. Values shown are from a single, representative experiment and are the mean ± SEM of six independent cultures for RC and three independent cultures for GC cells. The experiment was repeated and both experiments yielded comparable values for stathmin protein content. $^*P < 0.05$, RC versus GC.

al., 1989; Schubart et al., 1992; Hosoya et al., 1996; Rowlands et al., 1995], suggesting that reduction of stathmin may play a role in preventing proliferation and promoting terminal differentiation. This is consistent with our observations showing lower levels in the GC cells. Similarly, in primary fetal rat osteoblasts, stathmin mRNA is high in proliferating cells and decreased to undetectable amounts at terminal differentiation, defined as production of a mineralized extracellular matrix [Schubart et al., 1992]. The controls used in our study, fetal rat brain and adult rat brain, are at distinct states of maturation, and express different levels of stathmin. However, these cells are not from the same animal, nor from the same tissue, raising the concern that other aspects of physiology may have modulating effects on the expression of this protein. In our study, the RC and GC cells were obtained from the same rats and from the same tissue. In vivo, RC cells will ultimately differentiate and mature into GC cells; moreover, RC cells can be induced to acquire a GC phenotype in vitro [Schwartz et al., 1995; Schwartz et al., 1998b; Schwartz et al., 1998c]. Thus, the differential expression of stathmin in these cells under basal conditions strongly supports this hypothesis.

Although the amount of stathmin in costochondral chondrocyte cultures may be low when compared to other cells and its role is speculative, its expression and production agrees with that predicted in other cells. Others have suggested that stathmin may serve as a marker regarding the state of differentiation of cells in culture [Amat et al., 1991; Mistry and Atweh, 1999]. Our results indicate this is the case and suggest that stathmin may be a marker of differentiation of cells in vivo as well.

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