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Perinuclear Localization of an Intracellular Binding Protein Related to the Fibroblast Growth Factor (FGF) Receptor 1 Is Temporally Associated with the Nuclear Trafficking of FGF-2 in Proliferating Epiphyseal Growth Plate Chondrocytes*

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) is a potent autocrine mitogen for fetal epiphyseal growth plate chondrocytes and exhibits a transient nuclear translocation during G_1 of the cell cycle. We have characterized an intracellular binding protein (FGFBP) for FGF-2 that undergoes a juxtanuclear localization coincident with the nuclear translocation of the growth factor. Chondrocytes were isolated from the proliferative zone of the ovine fetal proximal tibial growth plate at 50–130 days gestation by collagenase digestion and were maintained in monolayer at early passage number. Cells were growth restricted by serum starvation for 48 h, and the synchronized culture was restarted into the cell cycle in the presence of 2% FBS. Cells were removed between 4–26 h of incubation, and fractions representing the plasma membrane, cytoplasm, nuclear membrane, and nuclear contents were separated by differential centrifugation. FGFBPs were separated using FGF-2 affinity chromatography. Ligand blot analysis using ^{125}I-labeled FGF-2 showed that a FGFBP of 46–48 kDa (represented by a double band) was present on the nuclear membrane at mid to late G_1, and Western blot showed this to be immunologically related to a part of the extracellular domain of the high affinity FGF receptor 1 (FGFR1). Immunocytochemistry with intact cell cultures showed that this protein underwent a juxtanuclear distribution through mid to late G_1. Immunoprecipitation was performed to monitor newly synthesized FGFR1 migration throughout the cell cycle. Synchronized cells were cultured in medium containing ^{35}S-labeled methionine/cysteine, and the cellular compartments were separated before immunoprecipitation using an antibody raised against the extracellular domain of FGFR1. Newly synthesized FGFR1-related proteins appeared throughout G_1 and migrated multidirectionally within the cell; intact receptor of 125–145 kDa accumulated at the plasma membrane, while both intact receptor and truncated FGFR1 of 46–48 kDa were detected on the nuclear membrane, but not within the nucleus. Cells were incubated with protamine sulfate to prevent the binding of endogenous, cell membrane-associated FGF-2 to high affinity FGFRs and their subsequent internalization. This did not alter the juxtanuclear accumulation of truncated FGFR1 in late G_1, suggesting that this was not derived from the plasma membrane. The truncated FGFR1 may mediate the nuclear translocation of FGF-2 during late G_1. (*Endocrinology* **137:** 5078–5089, 1996)

THE FIBROBLAST growth factor (FGF) family consists of nine structurally related peptides exhibiting 30–55% sequence homology (1), with a broad spectrum of biological activities, including the initiation of cell replication, morphogenic induction, chemotaxis, and the control of cell differentiation during embryonic and fetal development, compensatory organ growth, and wound healing (2). The FGFs interact with at least four related types of high affinity cell membrane receptors, designated FGFR1–R4, which are members of the tyrosine kinase gene family and activate the Ras protein signaling pathway subsequent to FGF binding, dimerization, and *trans*-phosphorylation (1, 3). However, cell cycle-related nuclear localization of FGF-1 (acidic), FGF-2 (basic), and FGF-3 (int-2) has also been reported in several cell types (4–10), by which the growth factors may exert direct transcriptional effects on nuclear or nucleolar genes (4, 11, 12). This may form an alternate or complimentary pathway(s) of FGF signaling applicable to its effects on mitogenesis and/or differentiation. We previously reported a transient nuclear translocation of FGF-2 within isolated epiphyseal growth plate chondrocytes from the ovine fetus, a cell type in which FGF-2 acts as an autocrine mitogen (13), at the end of G_1 of the cell cycle of replication (8). This was shown to represent at least in part newly synthesized FGF-2 directed toward the nucleus without prior secretion and subsequent internalization by the cell and appeared unrelated to the ability of endogenous externalized FGF-2 to act as an autocrine mitogenic signal.

Putative nuclear localization sequences have been described in FGF-1, -2, and -3 (14–17). FGF-2 can exist in at least four molecular mass forms that result from the use of alternative translation initiation codons (18), with larger forms between 22.5–24.2 kDa being preferentially recovered from the nucleus (15, 19). This implies the presence of specific nuclear recognition sequences in the amino-terminals of these peptides. As exogenous FGF-2 of 18 kDa can also be

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recovered from the nucleolus of endothelial cells or NIH-3T3 fibroblasts (4), it is possible that more than one nuclear recognition sequence exists in FGF-2. By analogy, FGF-3 has been shown to contain two nuclear localization sequences, both of which are necessary for optimal nuclear import (16). This may explain why mutagenesis of individual putative nuclear localization domains in FGF-1 and FGF-2 has not always prevented nuclear localization (20, 21).

The purpose of this study was to identify possible intracellular binding proteins for FGF-2 (FGFBP) that might be involved in the cell cycle-dependent nuclear localization of FGF-2 by isolated chondrocytes of the epiphyseal growth plate. Our findings suggest that a binding protein immunologically related to the extracellular domain of the FGFR1 receptor is associated temporally with the nuclear translocation of FGF-2. This protein does not derive predominantly from intact receptor internalized from the plasma membrane and may represent a distinct nuclear traffic route for intracrine-acting FGF-2.

Materials and Methods

Reagents

Recombinant human (h) FGF-2 was purchased from Upstate Biotechnology, Inc. (UBI; Lake Placid, NY), and a rabbit polyclonal antibody (Ab 773) raised against the 1–24 synthetic fragment of human FGF-2 was kindly provided by Dr. A. Baird, Scripps Research Institute (La Jolla, CA). The antibody demonstrates less than 1% cross-reactivity with FGF-1, FGF-3 (int-2), FGF-4 (hst/ks), FGF-5, FGF-6, and FGF-7. Cross-reactivity with FGF-8 and FGF-9 is not known. However, each of these peptides shows low sequence homology with the peptide fragment of FGF-2 used as the antigen (22). The same FGF-2 antibody was found to block the mitogenic actions of exogenous or endogenous FGF-2 on ovine growth plate chondrocytes *in vitro* (13). A polyclonal antiserum raised in rabbits against residues 119–144, representing a deduced portion of the acidic box region of the chicken FGF receptor (FGFR) extracellular domain, was obtained from UBI, as was the immunizing peptide for use as a competitive inhibitory agent during Western blot or immunoprecipitation. A polyclonal antibody raised in rabbit against residues 249–255 of the external juxtamembrane domain of human FGFR1 (Ab 6) was kindly provided by Dr. A. Baird (23), whereas a murine monoclonal antibody raised against the intracellular portion of the human FGFR1 (amino acid sequence 668–823, carboxyl-tail) was purchased from Transduction Laboratories (Lexington, KY). The intracellular domain sequence of FGFR1 against which the antibody was raised shows greater than 90% amino acid homology with FGFR2 and FGFR3 species (3).

Plastic tissue culture flasks (25 and 75 cm^2; Costar, Cambridge, MA), Triton-X, paraformaldehyde, and Ultralok centrifuge tubes (Nalgene) were purchased from Fisher Scientific (Whitby, Canada). Hanks' Buffered Salts Solution and DMEM (25 mM glucose, with and without methionine/cysteine) were obtained from Life Technologies (Burlington, Canada). FBS, trypsin-EDTA, penicillin/streptomycin, fungizone, and [^{3}H]thymidine (SA, 20 Ci/mmol) were acquired from ICN Biomedicals (Mississauga, Canada). Glutaraldehyde, 3–3′ diaminobenzidine tablets, Dulbecco's PBS, aprotinin, protamine sulfate, phenylmethylsulfonylfluoride, BSA, colorimetric acid phosphatase detection kit, goat antirabbit or antimouse IgGs (whole molecule) biotin conjugate, ExtrAvidin peroxidase, and Kodak X-Omat film were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was obtained from Boehringer Ingelheim (Canada) (Dorval, Canada), normal goat serum was obtained from Vector Laboratories (Burlingame, CA), and heparin-Sepharose and protein A-Sepharose were acquired from Pharmacia Biotech (Baie d'Urfé, Canada). Lab-Tek chamber slides (eight-well; Nunc, Inc., Napierville, IL) were purchased from VWR Scientific, (London, Canada), mol wt standards (low range) for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA), and Centricon microconcentrators were purchased from Amicon (Danvers, MA). [^{125}I]FGF-2 (SA, 1000 Ci/mmol) and Pro-Mix cell-labeling [^{35}S]methionine/cysteine (1000 Ci/mmol) were purchased from Amersham International (Mississauga, Canada), and Hoechst fluorochrome 33258 was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Chondrocyte isolation and tissue culture

Pregnant sheep of mixed breeds were killed with sodium pentobarbitone (Euthanyl, MTC Pharmaceuticals, Mississauga, Canada) between days 50–130 gestation (term = 145 days), and the fetuses were delivered. All experiments were conducted with prior approval of the ethical committees of the Lawson Research Institute and the University of Western Ontario in accordance with guidelines established by the Canadian Council for Animal Care. Cartilage was dissected from epiphyseal growth plate of the proximal tibia, and the chondrocytes were isolated after digestion of the matrix with 0.2% (wt/vol) collagenase, as previously described (24). Chondrocytes were plated in 75-cm^2 plastic culture flasks in DMEM supplemented with 10% FBS (vol/vol), penicillin (100 μU/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). Chondrocyte cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2-95% air for 2–3 days before use in experiments between passages 3–6. Cultures were visually monitored to ensure adherence of chondrocytes with flattened morphology to the culture flasks. Cultures with evidence of rounded or free floating cells were omitted.

Chondrocytes were grown in culture flasks with 10 ml DMEM-10% FBS (vol/vol) for 2–3 days until 80% confluent before being growth restricted by exposure to medium with 0.1% FBS (vol/vol) for 48 h. Entry into the proliferative cycle was initiated by the addition of fresh DMEM containing 2% FBS (vol/vol) for 4–26 h. Cultures were washed with PBS, and 2 ml homogenization buffer were added (20 mM Tris containing 0.33 M sucrose, 2 mM EDTA, and 2 mM phenylmethylsulfonylfluoride, pH 7.5). Cells were harvested by manually scraping them into the homogenization buffer within each flask, and the samples were removed and homogenized by hand with 10 strokes of a pestle before centrifugation at 2500 rpm for 10 min at 4 C. The pellet was considered to represent a crude nuclear fraction, whereas the supernatant was considered to represent a mixture of cytoplasm and plasma membranes. Nuclear or cytoplasmic fractions were each suspended in homogenization buffer further supplemented with 0.5 mM EGTA, 0.3% aprotinin (vol/vol), and 1% Nonidet P-40 (vol/vol) to a total volume of 2 ml and sonicated (three times, 10 sec, 30 Hz). Samples were then centrifuged in Ultralok tubes at 41,000 rpm in a Beckman ultracentrifuge (Beckman, Palo Alto, CA) for 30 min at 4 C. The observed pellet was considered to be either the plasma or nuclear membrane portion and was resuspended in 2.0 ml fresh homogenization buffer. The supernatants, representing either cytoplasm or nuclear contents, were saved after removal. All samples were stored at −70 C until further analysis. The efficiency of separation of the nuclear fractions was assessed by the presence of acid phosphatase in the nuclear membrane and contents, compared to total cell lysate, for cells isolated after 18 h of incubation. Approximately 13% acid phosphatase activity was associated with the nuclear fractions.

Separation of FGF-2 binding species by affinity chromatography

Affinity chromatography was performed at 4 C to isolate FGFBP species. A heparin-Sepharose affinity column was washed thoroughly with PBS and coated with 0.5% (wt/vol) BSA and 0.1% (wt/vol) sodium azide for 24 h to block nonspecific growth factor binding. FGF-2 (5 μg) was loaded onto the column and allowed to circulate for 18–24 h at a flow rate of 1 ml/min. The column was washed with PBS overnight before loading nuclear or cytoplasmic fractions pooled from four culture flasks. Samples were allowed to circulate for 24 h before further washing with PBS for 6 h and elution from the column using 1-ml volumes of increasing concentrations of NaCl in PBS (0.6–2.0 M). A 2.0-M concentration of NaCl has been demonstrated previously to completely elute FGF-2 from heparin (25). Eluted fractions were concentrated using Centricon microconcentrators before protein analysis.

Ligand blot analysis

Proteins eluted within fractions from heparin-Sepharose affinity columns were separated by SDS-PAGE under nonreducing conditions.

These samples were run in duplicate for identification by either protein staining or ligand blot analysis. Briefly, volumes of column fractions were mixed with modified electrophoresis sample buffer (26) in a 4:1 ratio and separated on 8% SDS-PAGE, and proteins were visualized within the dried gels by incubation overnight with either silver stain (Bio-Rad) or Coomassie blue stain. The gels were destained for approximately 3 h in 5% methanol (vol/vol) containing 7.5% acetic acid (vol/vol). Parallel samples separated by 8% SDS-PAGE using a Bio-Rad mini cell (Mini-Protean II Cell) at 200 mV for approximately 30 min were used to detect FGF-2-binding species by ligand blot analysis, as described previously (27). After separation, proteins were transferred onto nitrocellulose membranes using an electrophoretic blotting apparatus (Bio-Rad Transblot Cell, model 200/20; 200 mA for approximately 30 min to 1 h) at 4 C in prechilled buffer (25 mM Tris and 192 mM glycine; 20% methanol, vol/vol). The membranes were washed with prechilled buffers in the following order: 1) Tris-saline buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.4) containing 0.5 mg/ml sodium azide and 0.3% (vol/vol) Nonidet P-40, 30 min; 2) Tris-saline buffer with 1% (wt/vol) fatty acid-free BSA, 2 h; and 3) Tris-saline buffer with 0.1% Tween-20, twice, 20 min, followed by incubation in 10 ml Tris-saline buffer containing 1% (wt/vol) BSA and 0.1% (vol/vol) Tween-20 together with 400,000 cpm ^{125}I-labeled FGF-2 for 18 h at 4 C on an orbital shaker. After these incubations, membranes were thoroughly washed with Tris-saline buffer, air-dried, and then exposed to x-ray film (Kodak X-Omat) at −70 C in a cassette containing intensifying screens for 3–7 days. The specificity of FGF-2 binding to molecular species eluted from the affinity chromatography columns was determined by the addition of excess unlabeled FGF-2 (0.25 μg/ml) together with radiolabeled FGF-2 during incubation with duplicate sample lanes.

Western blot analysis

Western immunoblots were performed on protein samples separated by 8% SDS-PAGE and transferred to nitrocellulose membranes, as described above. Membranes were initially washed for 30 min in 10 mM Tris-HCl containing 0.15 M NaCl, 0.3% Nonidet P-40 (vol/vol), and 0.5 mg/ml sodium azide, pH 7.4. Nonspecific binding was reduced by incubation in Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing 0.05% Tween-20 (vol/vol; TTBS) supplemented with 4% BSA (wt/vol) for 30 min before washing three times with TTBS only (10 min/wash). All washes were performed at 23 C on an orbital shaker. The membranes were then incubated for 18–20 h at 4 C on an orbital shaker in TTBS containing 1% BSA (wt/vol) and one of the following primary antibodies: anti-FGFR1 receptor [polyclonal, raised in rabbits against a peptide sequence corresponding to residues 119–144 of the deduced complementary DNA (cDNA) of the chicken FGFR, acid box] diluted 1:250, anti-FGFR1 receptor (mouse monoclonal raised against a fragment of the human receptor cytoplasmic domain corresponding to amino acids 668–823 of the mature protein, carboxyl-tail) diluted 1:250, Ab 6 (rabbit polyclonal anti-FGFR1 antibody raised against a synthetic peptide representing residues 249–255 of the external juxtamembrane domain) diluted 1:60, or Ab 773 (rabbit polyclonal anti-FGF-2 antibody raised against the 1–24 synthetic fragment of human FGF-2) diluted 1:250. The membranes underwent the remaining washes and incubations at room temperature on the orbital shaker [three times, 10 min each time, in TTBS; antirabbit or antimouse IgG (whole molecule) biotin conjugates diluted 1:30 in 1% BSA (wt/vol)-TTBS for 2 h; three times, 10 min each time, in TTBS; ExtrAvidin diluted 1:30 in PBS for 1 h; and three times, 10 min each time, in TTBS]. Proteins were visualized by immersing the nitrocellulose membrane in diaminobenzidine [one tablet dissolved in 15 ml 50 mM Tris-HCl filtered through Whatman 4 filter paper (Whatman, Clifton, NJ)] and the subsequent addition of 10-μl volumes of 3% H_2O_2 (vol/vol) until immunoreactive species had reached their maximal staining intensity. The reaction was then quenched by incubation in 50 mM Tris-HCl (pH 7.5) overnight before air-drying.

Immunoprecipitation of FGFR species

Newly synthesized FGFR species were immunoprecipitated from the various cellular compartments and quantified at various times throughout the cell cycle. Chondrocytes were growth restricted before culture through the cell cycle, as described above. Cells were grown in methionine/cysteine-free DMEM supplemented with 60 μCi Pro-Mix cell-labeling [^{35}S]methionine/cysteine to each 75-cm^2 flask for the length of each incubation period (4–26 h) in the presence or absence of protamine sulfate (10 μg/ml). An aliquot of the cells in homogenization buffer was saved for analysis of protein content by the Lowry method (28), and the remainder was used to separate nuclear membrane, nuclear content, plasma membrane, and cytoplasmic fractions as described. Immunoprecipitation was performed on each using a polyclonal antibody raised against the extracellular domain (acidic box) of the chicken FGFR (UBI). A volume of each cellular fraction corresponding to 3.5×10^5 cells (~600 μl) was incubated for 2 h at 10 C on an orbital shaker with 10 μl of the antibody (1:60 final dilution). Immune complexes were precipitated by incubation with protein A-Sepharose beads for 2 h. Samples were centrifuged at 6500 rpm for 10 min at 23 C, the supernatants were aspirated, and the pellets were washed twice with PBS (pH 7.4) and twice with PBS containing 0.5 M NaCl. Radiolabeled proteins were quantified by liquid scintillation counting and were visualized by separation on 8% SDS-PAGE gels, followed by autoradiography for 7 days. Nonspecific binding was determined by the addition of the synthetic receptor fragment (10 μg/ml), which served as the antigen for the antibody, together with the antibody before the addition of protein A-Sepharose.

Immunocytochemical localization of FGF-2 and FGFR

Chondrocytes were cultured to confluency in eight-well Lab-Tek chamber slides before being growth restricted and restarted into the cell cycle as described above. Cells were fixed for histology between 4–28 h into the cell cycle by removal of the culture medium, washing briefly with PBS, and adding 4% paraformaldehyde (wt/vol)-0.2% glutaraldehyde (vol/vol) for 30 min. Fixative was removed with PBS, and FGF-2 and FGFR species were visualized by immunocytochemistry using the avidin-biotin complex method, as described by us previously (8). The primary polyclonal antibodies used were the rabbit anti-FGF-2 antibody (Ab 773) and a polyclonal antibody raised against the extracellular domain (acidic box) of the chicken FGFR (UBI); these were used at final concentrations of 1:1000 and 1:250, respectively. Positive staining was assessed by light microscopy. The specificity of staining was verified by several criteria. Immunostaining was absent when 1) nonimmune, protein A-Sepharose-purified rabbit IgG was substituted for the primary antibody; 2) the biotinylated second antibody was omitted; and 3) the primary antibody was preincubated for 24 h at 4 C with either 240 nM FGF-2 (Ab 773) or 10 μg/ml FGFR antigenic peptide (FGFR antibody) in a siliconized polypropylene tube before incubation on the slide.

Mitogenic stimulation of cell cultures

Monolayer cultures of chondrocytes were synchronized in the cell cycle and incubated for 14.5 h in serum-free medium alone or in serum-free medium containing 2% FBS or 10 ng/ml FGF-2 to determine the effects of mitogenic stimulation on the presence of the intracellular FGFBP species. Proteins from crude nuclear and cytoplasmic fractions were separated on 8% SDS-PAGE, and nitrocellulose blots containing transferred proteins were incubated with 400,000 cpm [^{125}I]FGF-2 by ligand blot analysis. Autoradiography was performed by exposing the blots to x-ray film for 3–6 days.

Chondrocyte cell cycle kinetics

Chondrocytes were plated into 6-well culture dishes at a density of 20,000 cells/well in 1 ml DMEM-10% (vol/vol) FBS and were cultured for 3–4 days until approximately 80% confluent. Cells were then growth restricted by incubation in 1 ml DMEM-0.1% (vol/vol) FBS for 48 h before the medium was replaced with 1 ml DMEM supplemented with 2% FBS. Cultures were terminated at 2-h intervals for up to 44 h after the addition of 1 μCi [^{3}H]thymidine to each well in 20 μl medium for the final 2 h of incubation. In some experiments the culture medium was supplemented with FGF-2 (0.56 nM) with or without protamine sulfate (10 μg/ml) and incubated for 20 h before the addition of [^{3}H]thymidine. The cells were allowed to continue growing for an additional 4 h before being washed with PBS (pH 7.4) to remove unincorporated isotope, and DNA and protein were precipitated with 1 ml ice-cold trichloroacetic acid (10%, wt/vol) and solubilized by overnight incubation at 37 C with 1 M sodium hydroxide (500 μl/well). Isotope incorporation was measured by liquid scintillation counting and expressed as disintegrations

per min/μg DNA. The DNA content of each culture well was measured by fluorometry, using Hoechst fluorochrome 33258, as described previously (24). The length of the G_1 phase of the proliferative cycle was determined from a sharp peak of [^{3}H]thymidine incorporation during S phase.

Statistical analysis

All experiments were performed at least three times. Results are expressed as the mean ± SE. Statistical differences between mean values were calculated using ANOVA and the Bonferroni multiple comparisons test.

Results

Subcellular localization of FGF-2 immunoreactivity

Chondrocytes that had been growth restricted by incubation in DMEM containing 0.1% FBS were restarted into the cell cycle by exposure to 2% FBS. The monolayers were incubated for periods between 4–44 h and labeled with [^{3}H]thymidine during the final 2 h. A sharp peak of radiothymidine incorporation was seen after 20 and 22 h of incubation, indicating the entry of the majority of cells into S phase of the cell cycle (Fig. 1). Rounded cells undergoing mitosis were seen by light microscopy after approximately 26 h, and a smaller peak of thymidine incorporation was seen after 42 h of incubation, which probably represented the S phase of a second cycle of proliferation. Cells restarted into the cell cycle were cultured for between 4–28 h on chamber slides before fixation and the visualization of FGF-2 by immunocytochemistry (Fig. 2, A–D). Chondrocyte monolayers appeared immunonegative for FGF-2 after 12 h of incubation (Fig. 2A); however, diffuse cytoplasmic staining was apparent by 18 h of incubation (not shown). After 20 h of incubation (Fig. 2B), FGF-2 was visualized within the majority of cell nuclei, but staining had largely disappeared from the nuclei by 22 h (Fig. 2C) and from both cellular compartments by 24 h. The nuclear localization of FGF-2 immunoreactivity was, therefore, temporally associated with the G_1/S phase boundary.

Visualization of FGF-binding species

To identify possible binding proteins responsible for delivering FGF-2 to the nuclear membrane by late G_1 phase, cytoplasmic/plasma membrane or nuclear fractions were separated from cells synchronized in culture between 4–22 h of the cell cycle. Each fraction was extracted on an FGF-2-heparin-Sepharose affinity column, and proteins were eluted with increasing salt concentrations before SDS-PAGE followed by ligand blot analysis with ^{125}I-labeled FGF-2. In both the cytoplasmic/plasma membrane and nuclear fractions from synchronized cells, FGF-binding species were eluted between 1.0–1.6 M NaCl (Fig. 3). Within the cytoplasm/plasma membrane, FGF-binding species of approximately 18–20, 36–40, 46–48, and 80–145 kDa were seen at 4 h (not shown), 12 h [Fig. 4 (−)], 18 h (not shown), and 20 h (Fig. 3a). Protein species were difficult to detect by ligand analysis at 14 and 16 h (not shown). In nuclear fractions, the 46- to 48-kDa form predominated at 4, 12, 14, 16, 18 (not shown), 20 (Fig. 3c), and 22 h [Fig. 4 (−)]. The larger protein species were present in nuclear fractions, but bound [^{125}I]FGF-2 differentially. A protein species evident at 18–20 kDa could represent FGF-2 initially bound to the high affinity binding sites of the receptor species that dissociated on SDS-PAGE and dimerized to the radiolabeled growth factor. Binding of radiolabeled FGF-2 was abolished when nitrocellulose filters containing 12-h cytoplasmic or 22-h nuclear transferred proteins were incubated in the presence of excess unlabeled FGF-2 (Fig. 4). However, considerable amounts of other proteins were also eluted from the FGF-2-heparin affinity column when analyzed by silver staining (Fig. 3, b and d).

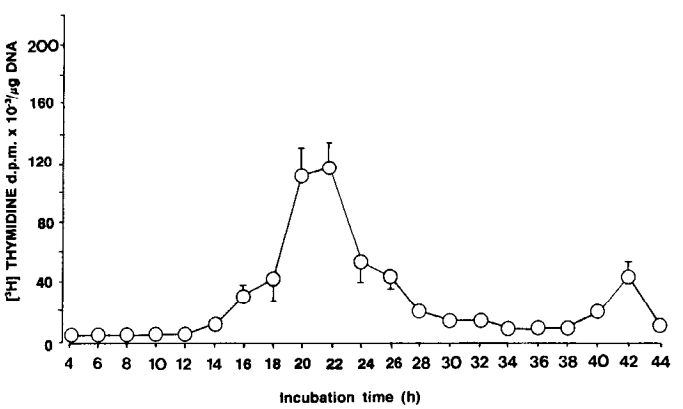

FIG. 1. Incorporation of [^{3}H]thymidine into DNA in monolayer cultures of synchronized growth plate chondrocytes incubated between 4–44 h. For each time point, [^{3}H]thymidine was present for the final 2 h of incubation. Figures represent the mean ± sem for six replicate incubations.

Immunological identification of FGF-binding species

Western blot analysis of 22-h nuclear samples (Fig. 5, lanes C–G) confirmed that an intermediate protein species of 46–48 kDa that binds FGF-2 by ligand blot analysis (Fig. 5, lane A) is immunologically related to the FGFR1. Incubation of blots with an antibody directed against the intracellular domain of FGFR1 showed that FGF-binding species of 80 kDa or more present in plasma membrane fractions of unsynchronized chondrocyte cultures represented FGFRs (Fig. 5, lane B), which could also be visualized in 22-h nuclear fractions by ligand blotting with [^{125}I]FGF-2 (Fig. 5, lane A). Antibody against the intracellular domain of FGFR1 failed to recognize the smaller mol wt FGF-binding species in nuclear (Fig. 5, lane C), plasma membrane (Fig. 5, lane B), or cytoplasmic fractions (not shown). However, Western blot analysis of the same fractions using antibodies raised against the acidic box region in the extracellular domain of FGFR1 or against the juxtamembrane region of the extracellular domain of human FGFR1 recognized a double protein band of approximately 46–48 kDa (Fig. 5, lanes D and E). Staining was abolished when the antibody against the acidic box region of the receptor was preincubated with FGFR1-immunizing peptide before incubation of the nitrocellulose filter (Fig. 5, lane F). Nuclear proteins immunoprecipitated using the acid box antibody and detected by Western blot analysis using Ab 6 (juxtamembrane) demonstrated greater resolution and recovery of intact and intermediate (46–48 kDa) receptor species (Fig. 5, lane G). No immunoreactive FGF-2 was detected after Western blot analysis in either cytoplasmic/plasma membrane or nuclear fractions, demonstrating that any endogenous FGF-2 associated with the FGF-binding

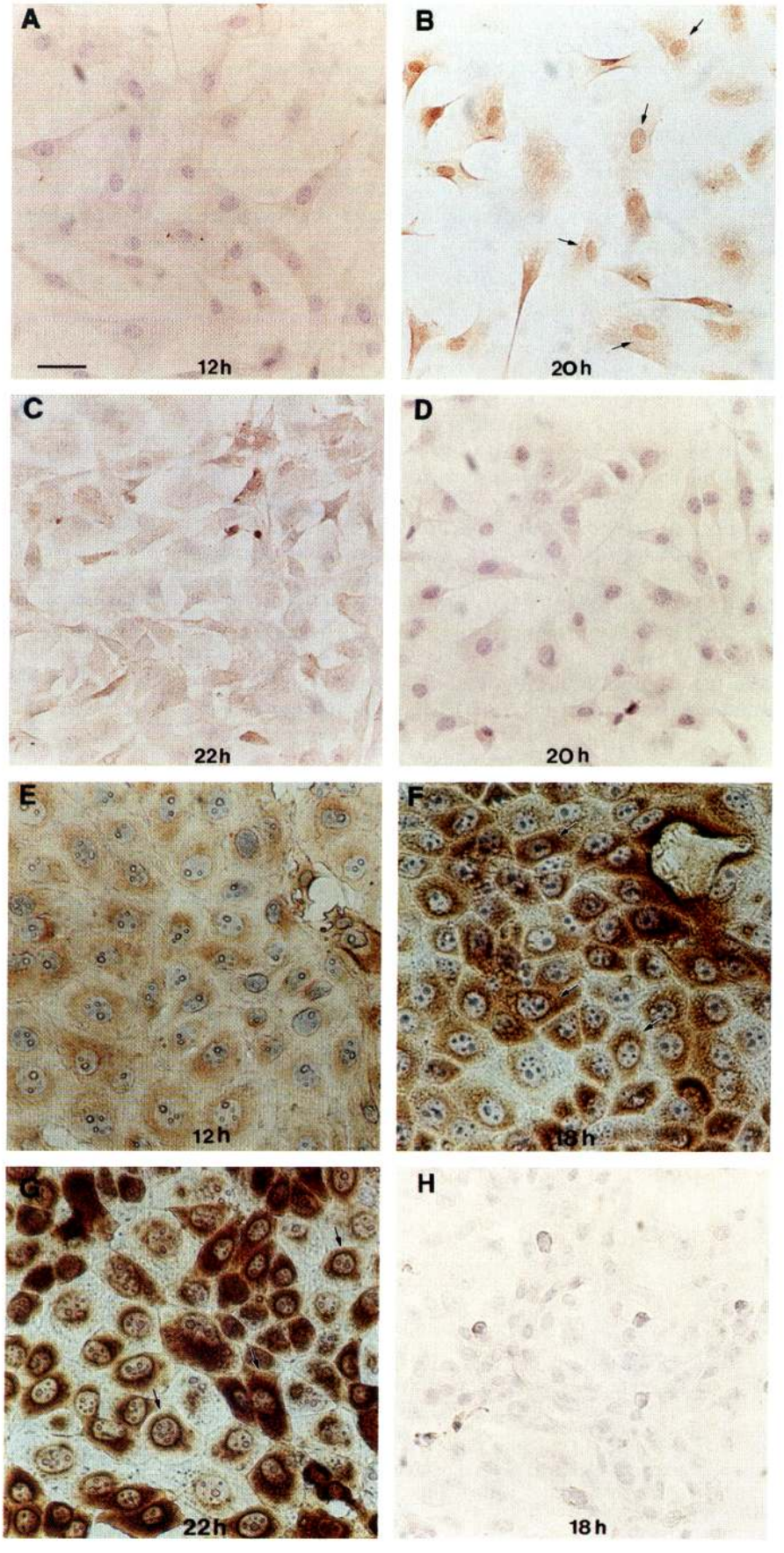

FIG. 2. Immunocytochemical localization of FGF-2 or FGFR-related peptides in monolayer cultures of ovine fetal growth plate chondrocytes. Synchronized cells were removed from culture 12, 18, 20, or 22 h after reentry into the cell cycle under a stimulus of 2% (vol/vol) FBS and fixed, and immunocytochemistry was performed using a primary antiserum against FGF-2 (A–D) or against the acidic box region of the extracellular domain of the FGFR (E–H). No staining for FGF-2 was seen at 12 h (A) or at other time points before 18 h, when a diffuse FGF-2 presence was seen in the cytoplasm (not shown). After 20 h of incubation (B), almost all cells demonstrated cytoplasmic staining together with strong nuclear staining (*arrows*). By 22 h, much of the nuclear staining had disappeared, and immunoreactive FGF-2 remained only in the cytoplasm (C). Preincubation of the primary antiserum with 240 nM FGF-2 before application to cells fixed after 20 h of incubation completely abolished the immunodetection of FGF-2 (D). Staining for FGFR-related peptides was seen within the cytoplasm of chondrocytes after 12 h of culture (E), and a strong perinuclear distribution (*arrows*) was seen between 18–22h (F and G). Preincubation of the primary antiserum with 10 μg/ml FGFR antigenic peptide before application to cells fixed after 18 h of incubation completely abolished the immunodetection of the FGFR-related species (H). The *scale bar* represents 10 μm.

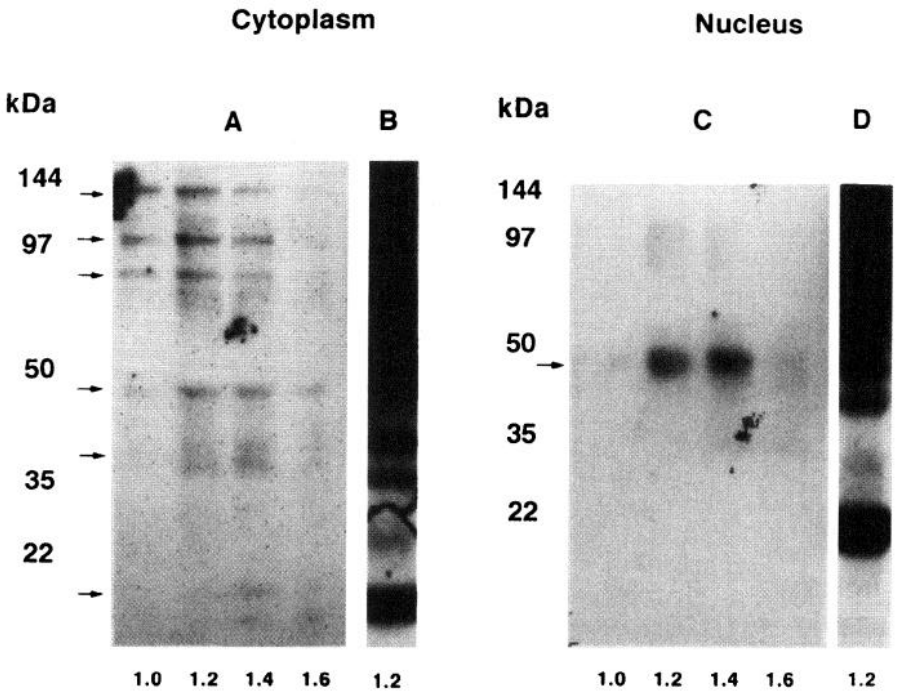

FIG. 3. Visualization of FGF-2-binding species in cytoplasmic/plasma membrane (A) or nuclear fractions (C) from synchronized cultures of growth plate chondrocytes after 20 h of incubation. Fractions were applied to a FGF-2-heparin-Sepharose column and eluted between 1.0–1.6 M NaCl, and the eluates were separated by 8% SDS-PAGE before transfer of the proteins to nitrocellulose membranes and ligand blotting with ^{125}I-labeled FGF-2. FGF-2-binding species of approximately 18–20, 36–40, 46–48, and 80–145 kDa were separated from the cytoplasmic fractions, and species of 46–48 kDa were separated from the nuclear fractions (*arrows*). Total protein eluted by 1.2 M NaCl from cytoplasmic (B) or nuclear (D) fractions was revealed by silver stain. Mol wt markers are shown to the *left*.

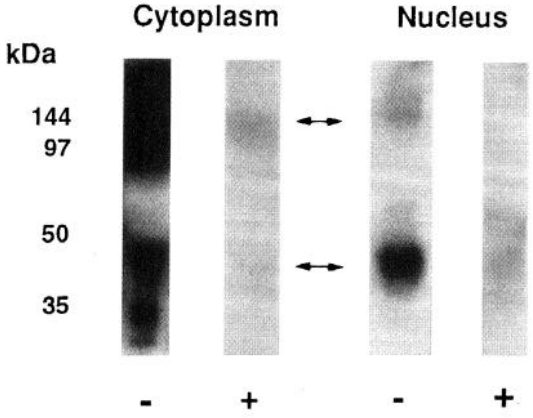

FIG. 4. Specific binding of FGF-2 to binding protein species demonstrated by ligand blot analysis of 22 h nuclear and 12 h cytoplasmic samples incubated with ^{125}I-labeled FGF-2 (−). Protein species of 80–145 and 46–48 kDa did not bind the radiolabel when incubated in the presence (+) of excess unlabeled FGF-2 (0.25 μg/ml). Mol wt markers are shown to the *left*.

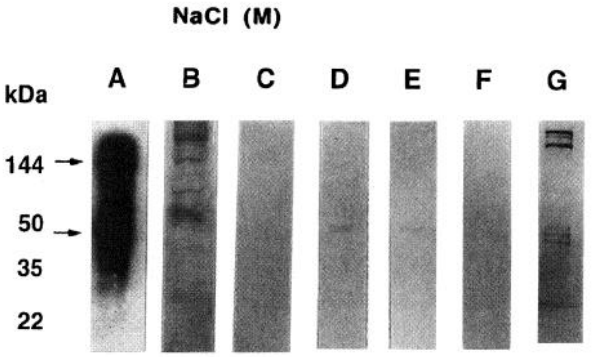

FIG. 5. Ligand blot analysis (A) or Western blot analysis (B–G) of 22 h nuclear samples to identify binding protein species. Ligand blot analysis (A) demonstrated protein species of 80–145 and 46–48 kDa that bind [^{125}I]FGF-2. Nuclear proteins were immunonegative when incubated with an antibody recognizing the internal C-terminus domain (C), which recognized larger mol wt receptor species in a cytoplasmic membrane control containing intact receptor (B). Antibodies raised against the external acid box domain (D) or the external juxtamembrane peptide sequence (E) were immunopositive for binding protein species of approximately 46–48 kDa. The juxtamembrane antibody was immunonegative when preabsorbed with excess FGFR1 peptide (F). Nuclear proteins immunoprecipitated with the acid box receptor antibody and visualized by Western blot analysis with the juxtamembrane receptor antibody demonstrated greater resolution and recovery of the large and intermediate protein species (G).

species was probably dissociated during affinity column elution or SDS-PAGE or was present in levels too low to detect by this method.

Cell cycle kinetics of radiolabeled FGFR-related proteins

Synchronized chondrocytes were incubated between 4–26 h with ^{35}S-labeled methionine/cysteine before preparation by ultrafiltration of cellular fractions representing the plasma membranes, cytoplasm, nuclear membranes, and nuclear contents. Each fraction was then immunoprecipitated with a primary antiserum raised against the extracellular acidic box region of the FGFR, and the associated radioactivity specifically bound was quantified by liquid scintillation counting. Newly synthesized FGFR-related proteins were detectable in the nuclear membranes in greatest abundance by 8 h of incubation (Fig. 6a). The amount of radiolabeled FGFR-related protein associated with the nuclear membranes plateaued at a half-maximal level throughout the remainder of G_1/S. Separation of the radiolabeled proteins on a represen-

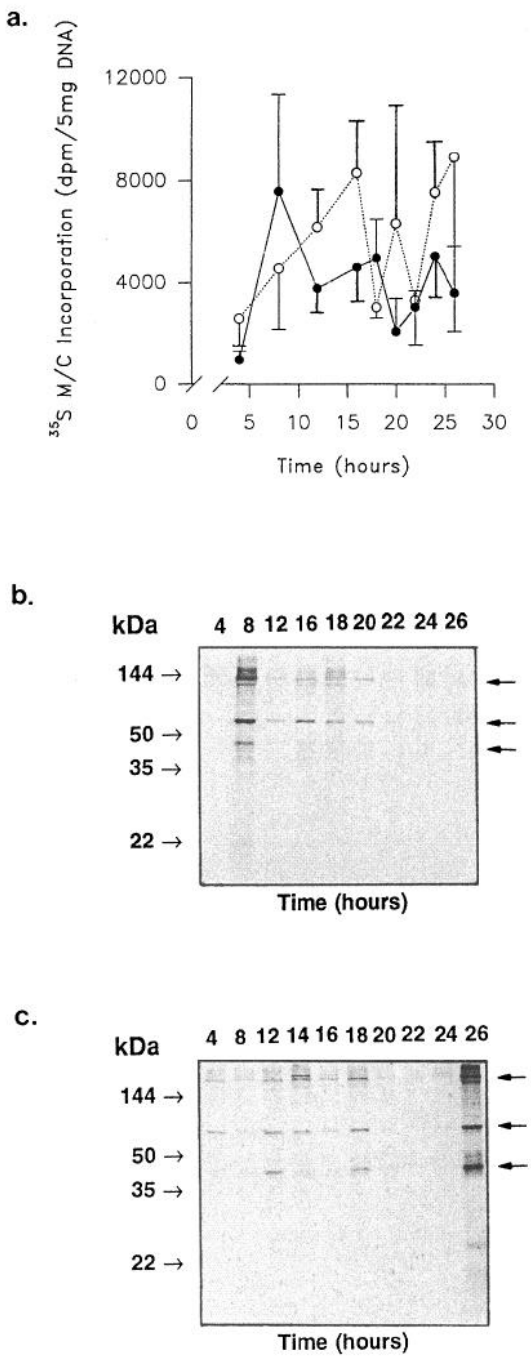

FIG. 6. Immunoprecipitation of newly synthesized FGFR1-related peptides after labeling with [^{35}S]methionine/cysteine (M/C) from the nuclear membranes and quantification by liquid scintillation counting (a). Values represent the mean ± SEM for three separate experiments. Radiolabeled immunoprecipitated species from the nuclear membranes of control (●) cultures or those incubated in the presence of 10 μg/ml protamine sulfate (○) were visualized by autoradiography after separation on SDS-PAGE (2 mg protein loaded/lane; mol wt markers to the *left*). An increase in control nuclear membrane-associated immunoreactivity was maximal by 8 h of the cell cycle and declined to half-maximal levels thereafter (a). The predominant protein species visualized (b), which decreased in abundance from 8–20 h, were approximately 120–145, 80, and 46–48 kDa (←). A peak of radiolabel activity in protamine sulfate cultures was observed at 16 h, followed by a second peak of activity at 26 h. Proteins of similar mol wt to controls were observed through mid to late G_1 and at 26 h by SDS-PAGE (c; ←).

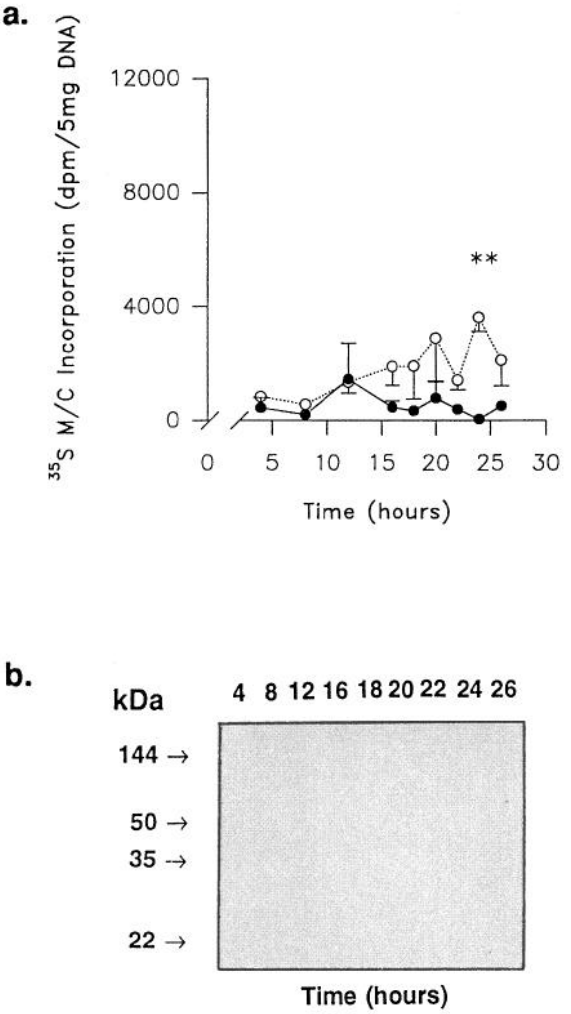

FIG. 7. Immunoprecipitation of newly synthesized FGFR1-related peptides after labeling with [^{35}S]methionine/cysteine (M/C) from nuclear contents and quantification by liquid scintillation counting (a). Values represent the mean ± SEM for three separate experiments of control (●) or protamine sulfate-incubated (○) cultures. Immunoprecipitated protein species (2 mg/lane) of control samples were separated by SDS-PAGE and visualized by autoradiography (mol wt markers to the *left*). Little FGFR-related material was recovered from the nuclear contents under either condition; a significant increase was observed at 24 h of incubation (**, $P < 0.01$). No protein species were visualized (b).

tative SDS-PAGE followed by autoradiography revealed that the most abundant FGFR-related species were of 46–48, 80, and greater than 140 kDa and were of greatest intensity at 8 h (Fig. 6b). These proteins decreased in abundance through mid to late G_1. Only small amounts of radiolabeled activity were detectable in the nuclear contents at any point of the cell cycle (Fig. 7a) and were not visualized by SDS-PAGE autoradiography (Fig. 7b). Immunoprecipitation of the plasma membrane fractions showed that the newly synthesized FGFR forms were present after only 4-h incubation and were most abundant at mid-G_1 (12–14 h), declining to levels observed at 4 h by late G_1/S (Fig. 8a). The most abundant molecular forms of FGFR protein in the plasma membrane fractions were of approximately 120–145 kDa (visualized from 4–18 h), although the 46- to 48-kDa and 80-kDa im-

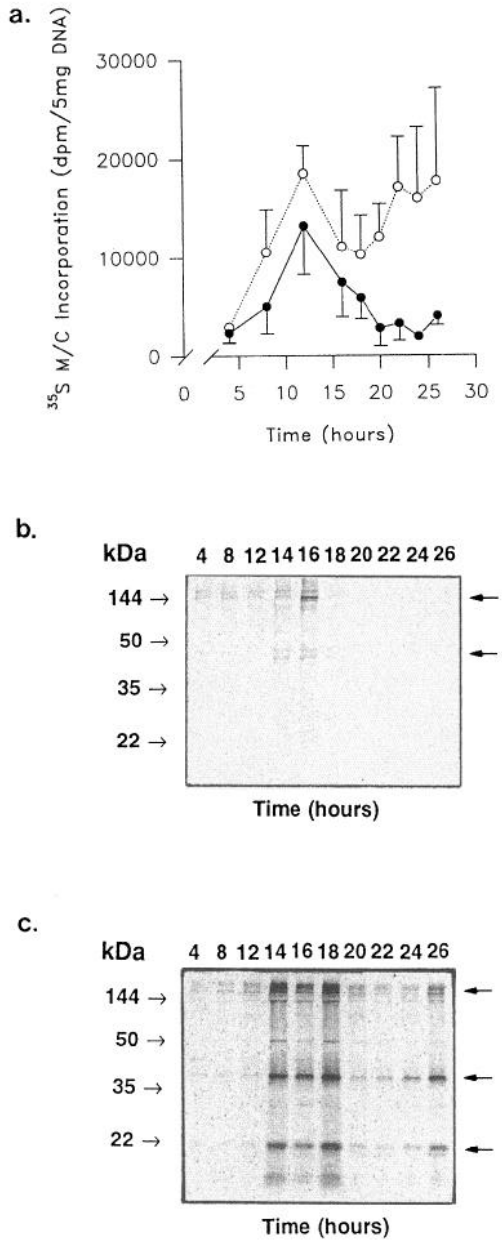

FIG. 8. Immunoprecipitation of newly synthesized FGFR1-related peptides after labeling with [^{35}S]methionine/cysteine (M/C) from the plasma membranes (a) of chondrocytes and quantification by liquid scintillation counting. Values represent the mean ± SEM for three separate experiments. Visualization by autoradiography of radiolabeled immunoprecipitated species from the plasma membranes after the incubation of cells in control conditions (b) or in the presence of protamine sulfate (10 μg/ml; c) and the separation of membrane proteins on SDS-PAGE (2 mg protein loaded/lane; mol wt markers to the *left*). An increase in plasma membrane-associated immunoreactivity was seen, which was maximal under control conditions (●) after mid-G_1 and subsequently declined (a). In cultures exposed to protamine sulfate (○), the FGFR-related immunoreactivity associated with the plasma membranes peaked at mid-G_1 and did not decline significantly thereafter. The predominant immunoreactive protein species visualized in control plasma membranes were of approximately 120–145 kDa (visualized throughout G_1), with minor bands of 46–48 kDa visible at mid to late G_1 (b; ←). In contrast, protamine sulfate plasma membranes demonstrated an increased abundance of these immunoreactive protein species through 26 h (maximal at mid to late G_1 and at 26 h). Additional species of approximately 18–24 kDa were also present (c; ←).

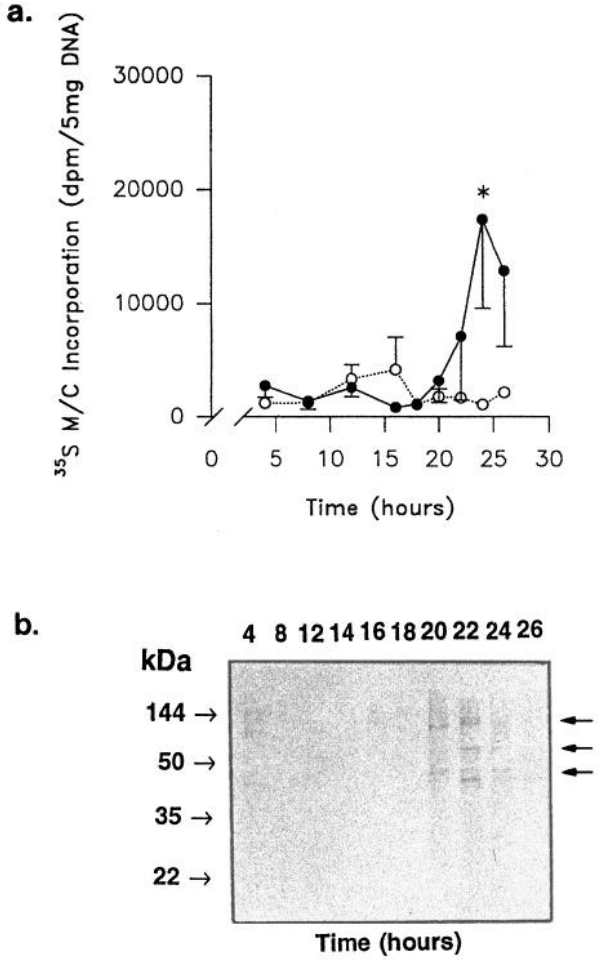

FIG. 9. a, Immunoprecipitation of newly synthesized FGFR1-related peptides after labeling with [^{35}S]methionine/cysteine from the cytoplasm of chondrocytes and quantification by liquid scintillation counting. Values represent the mean ± SEM for three separate experiments. b, Visualization by autoradiography of radiolabeled immunoprecipitated species from the cytoplasm after the incubation of cells under control conditions and the separation of membrane proteins on SDS-PAGE (2 mg protein loaded/lane; mol wt markers to the *left*). Cytoplasm-associated immunoreactivity under control conditions (●) was low until after entry of the cells into S phase from approximately 22 h (*, $P < 0.05$). In cultures exposed to protamine sulfate (○), the FGFR-related immunoreactivity associated with cytoplasm remained low throughout the cell cycle. During S phase, the predominant immunoreactive protein species visualized in control cultures were of approximately 120–145 kDa, with minor bands at 80 and 46–48 kDa (←).

munoreactive species were also visible at mid to late G_1 after SDS-PAGE (Fig. 8b). The decrease in FGFR proteins through late G_1/S phases at the plasma membrane was partially associated with an increased level of radioactivity within the cytoplasm at 20–24 h (Fig. 9a), and both 120- to 140-kDa and smaller forms of FGFR-related proteins were present in the cytoplasmic fractions at this time (Fig. 9b). Little receptor-related material was detected in the cytoplasm at earlier times and, thus, cannot account for all material dispersed from the plasma membrane. We previously described a soluble protein species of approximately 55–60 kDa representing a truncated portion of the FGFR1 in normal term ma-

ternal serum (29). A mechanism of release to the culture medium of solubilized receptor may contribute to the decrease observed at the cell membrane. The temporal and structural differences in the abundance of newly synthesized FGFR-related species throughout the chondrocyte cell cycle suggest a differential intracellular trafficking of various molecular forms.

Parallel experiments were performed in which chondrocytes were incubated in the presence of 10 μg/ml protamine sulfate to determine its effects on FGF-stimulated mitogenesis. Protamine sulfate has been demonstrated to inhibit mitogenic signaling by FGF-2 via cell membrane receptors and to prevent ligand-receptor internalization (30). Similar results were obtained in this study, as protamine sulfate prevented the increase in [^{3}H]thymidine incorporation into synchronized cultures of growth plate chondrocytes seen in response to exogenous FGF-2 (0.56 nM) and tended to decrease the basal DNA synthetic rate in control cultures (Fig. 10). Overall, exposure to protamine sulfate did not significantly alter the amounts of newly synthesized FGFR-related proteins recovered from either the nuclear membranes or nuclear contents throughout the cell cycle (Figs. 6a and 7a). However, the peak of activity at the nuclear membrane was delayed from 8 h until 16 h, and a significant increase in activity within the nucleus was determined at 24 h. The same protein species were visualized by SDS-PAGE analysis at the nuclear membrane in greatest abundance at mid to late G_1 and at 26 h (Fig. 6c). The presence of protamine sulfate prevented the rise in cytoplasmic FGFR-related proteins seen at midcycle, between 20–24 h of incubation (Fig. 9a), while causing an increased abundance at the cytoplasmic membrane (Fig. 8a); findings consistent with an inhibition of ligand-mediated receptor internalization. Receptor-related activity remained elevated over the control value throughout the 26 h incubation with protamine sulfate. FGFR-related species of 120–145, 80, 46–48, and 18–24 kDa were detected in plasma membranes after SDS-PAGE (Fig. 8c).

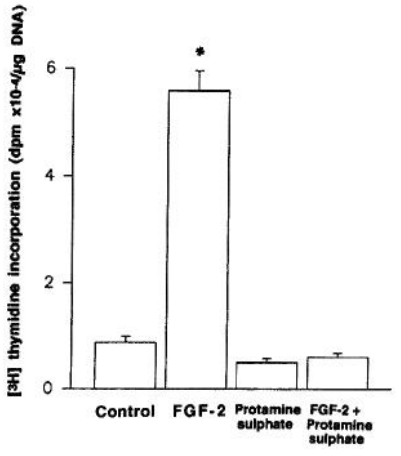

FIG. 10. Incorporation of [^{3}H]thymidine into DNA by isolated ovine fetal growth plate chondrocytes incubated for 24 h with or without exogenous FGF-2 (0.56 nM), protamine sulfate (10 μg/ml), or FGF-2 together with protamine sulfate. Figures represent the mean ± SEM for six replicate incubations within each of three representative experiments. *, $P < 0.001$ *vs.* control, *vs.* FGF, or *vs.* FGF plus protamine sulfate.

Subcellular distribution of FGFR-related species

Monolayer cultures of chondrocytes were fixed, and the presence of FGFR-related species was examined by immunocytochemistry, using a primary antibody recognizing the extracellular acid box domain of the receptor. Nonsynchronized cultures showed a differential staining pattern, with some cells demonstrating slight cytoplasmic and plasma membrane staining and others demonstrating dark perinuclear staining. Synchronized cultures (Fig. 2, E–G) demonstrated a cell cycle-related perinuclear distribution of FGFR-related species apparent from 12–22 h of incubation. Staining was abolished when the primary antibody was preabsorbed with FGFR-immunizing peptide before incubation with the cells (Fig. 2H).

Influence of mitogenic stimulants on the presence of FGFR-related species

Monolayer cultures of chondrocytes demonstrated a differential expression of FGFBP species depending upon the level of mitogenic stimulation (Fig. 11). A slight increase in abundance of the intermediate 46- to 48-kDa protein species was visualized by ligand blot analysis in nuclear fractions of cultures stimulated with FBS, whereas abundance was dramatically increased in the presence of FGF-2. Intact receptor and intermediate protein species were also visualized within the cytoplasm of these stimulated cultures (data not shown). Large protein species were not readily visible in nuclear fractions; however, smaller bands of 18–24 kDa were visible in both fractions, indicating the possible presence of FGF-2 within the samples, which could have dimerized with the radiolabeled FGF-2.

Discussion

These results show that high affinity FGFBP species immunologically related to the FGFR1 signaling receptor are present in isolated fetal growth plate chondrocytes and undergo a juxtanuclear localization through G_1 of the cell cycle.

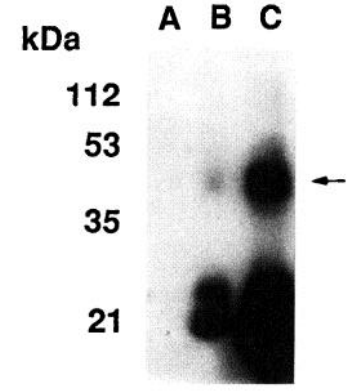

FIG. 11. Ligand blot analysis with [^{125}I]FGF-2 of nuclear fractions from chondrocyte cultures stimulated for 14.5 h with serum-free medium (A), serum-free medium containing 2% FBS (B), or serum-free medium containing 10 ng/ml FGF (C). The 46- to 48-kDa binding protein species within cultures increased in abundance with mitogenic stimulation (←). An increased abundance of proteins at 18–22 kDa was also visualized, which may be the dimerization of the radiolabel to FGF-2 within the fractions.

We have previously shown FGF-2 to act as an autocrine mitogen in this cell type, and that endogenous FGF-2 undergoes a nuclear translocation in late G_1 (8, 13). The FGFBPs partially characterized here may aid in the transport of FGF-2 to the nuclear membrane, although they do not appear to enter the nucleus themselves.

The intact FGFR1 cDNA predicts a protein with three Ig-like loops within the extracellular domain, the first and second separated by a region of 14 acidic amino acids referred to as the acidic box (3). The intracellular domain contains two tyrosine kinase sequences, and the entire glycosylated receptor molecule has a molecular size of approximately 150 kDa when separated by SDS-PAGE (3). Specific binding of FGF-2 to FGFR1 has been shown to be dependent on domains within the second and at least part of the third IgG-like loops (31). The FGF-binding species identified in this study associate with the nuclear membrane. Protein species of 120–145 kDa, corresponding to near full-length receptor, were recognized at the nuclear membrane by immunoprecipitation with an antibody recognizing the acid box region of the receptor. These species bound FGF-2 relatively poorly. The major receptor-related species localized to the nuclear membrane was of 46–48 kDa and possibly represents a truncated fragment of the extracellular domain of FGFR1. These were recognized by antisera directed against both the acidic box region and the juxtamembrane extracellular region, but not by an antibody directed against the carboxyl-terminus. The 46- to 48-kDa species showed strong and specific binding of radiolabeled FGF-2. The above properties would be compatible with a minimum structure for peptides of approximately 260 amino acids containing five possible residues available for N-linked glycosylation, assuming overall conservation of the structure of ovine FGFR1 compared to that reported for other species (3). An additional FGFR-related species of approximately 80 kDa was also found in association with the nuclear compartment by immunoprecipitation, but did not bind radiolabeled FGF-2 by ligand blot analysis.

The human FGFR1 gene contains at least 17 exons (31), and alternate exon splicing from the primary RNA transcript for FGFR1 can yield species of messenger RNA that translate to proteins lacking the first IgG-like domain and/or terminating short of the transmembranal region of the receptor (32, 33). Cross-linking of FGF-1 or FGF-2 to cell membrane receptors after the transfection of cells with either three Ig or two Ig receptor cDNA constructs has shown the existence of 145- and 125-kDa forms, respectively (34, 35). Receptor-related immunoprecipitable species of both sizes were separated here from plasma membranes of growth plate chondrocytes. A human cDNA has been isolated in which alternate exon usage results in a differing structure for the third Ig-like loop in the extracellular domain (33). This receptor form terminates short of the hydrophobic transmembranal region and has the capability to form a released soluble receptor (36) or to be theoretically retained within the cytoplasm as an intracellular FGFBP of the kind detected in this study. A complex of approximately 40–50 kDa has also been reported in human hepatoma cells and normal rat hepatocytes comprising the extracellular domain of a FGFR, including the first Ig-like loop, and an FGF (37). Similarly, Chinese hamster ovary cells transfected with a murine FGFR1 gene expressing two IgG loops and lacking the transmembrane and cytoplasmic sequences were found to secrete proteins of 45 kDa mol wt recognized by FGFR1 antibodies (38). Thus, the 46–48 kDa intracellular FGFBP found here may result from alternate exon usage of the FGFR1 gene.

An alternative explanation could be that the intracellular binding protein is derived from intact FGFR1 at the plasma membrane after internalization and selective proteolysis. The latter seems unlikely from the results of our temporal study of the appearance of intact receptor *vs.* intracellular binding protein throughout the cell cycle. Firstly, a newly synthesized, immunoprecipitable FGFBP of 46–48 kDa is present on the nuclear membranes of chondrocytes within 4 h of entry into G_1 phase, although the activity of this newly synthesized receptor was too low to be detected by SDS-PAGE autoradiography. A maximal accumulation was evident by 8 h. The intact receptor forms of 145 and 125 kDa are not detected on the plasma membrane in appreciable amounts until 8 h into the cell cycle and do not decline, suggestive of internalization, until after 12 h. The release of soluble receptor forms to the culture medium may also account for the decline in radioisotope activity at the cell surface. Further, incubation with protamine sulfate caused the maintenance of intact receptor at the plasma membrane in late G_1 phase and prevented its subsequent appearance in the cytoplasm. This would be consistent with a sequestration of endogenous FGF-2 by protamine sulfate, which prevents ligand-receptor interaction, and internalization of the complex. Consequently, protamine sulfate is able to prevent any increase in DNA synthesis by chondrocytes in the presence of exogenous FGF-2 and slightly decreases the DNA synthetic rate under basal conditions, possibly by the sequestration of endogenous growth factor. Despite the ability of protamine sulfate to alter the distribution of FGFR species between the plasma membrane and the cytoplasm, incubation in its presence did not alter the appearance of the 46- to 48-kDa FGFBP. This suggests that the latter is not derived primarily from the intact receptors at the plasma membrane subsequent to internalization and lysosomal proteolysis. However, an FGFBP of similar size was also found associated with the plasma membrane. Its relationship to the nuclear membrane form and whether this is derived from proteolytic cleavage of the intact receptor are not known.

The immunoprecipitation studies, therefore, suggest that FGFR1-related proteins are newly synthesized in G_1, and that once produced they migrate multidirectionally throughout the cell. The intact receptor is the predominant species appearing at the plasma membrane, but also undergoes a juxtanuclear translocation, as does a truncated form representing a portion of the extracellular domain. Transfection of 3T3 fibroblasts with a human FGFR1 cDNA representing the entire coding sequence resulted in a juxtanuclear localization of the receptor during G_1 phase of cell replication induced by exogenous FGF-1 (39). Similarly, breast epithelial cells transfected with full-length FGFR1 constructs expressed immunofluorescent staining at the plasma membrane and in cytoplasmic vesicles, which stained with strong intensity around the nucleus (40). It has been predicted in these same cells that FGFR3 localizes to the nucleus, and that this form

lacks the latter part of IgG loop 2 and the transmembrane region. This predicts a protein form that is mobile and may be secreted or remain as an intracellular transport mechanism.

The appearance of FGFR1 at the plasma membrane during G_1 phase coincides with the time at which immunoreactive FGF-2 has been detected at the cell membrane, possibly associated with cell membrane glycosaminoglycans (8). This suggests that the autocrine actions of FGF-2 on growth plate chondrocytes involve a temporal coordination of both ligand and receptor presence. If the intracellular FGFBP described here is involved with a nuclear localization of FGF-2, it is not clear whether this is endogenous FGF-2 that has been secreted by the cell and subsequently internalized in association with the plasma membrane receptors or newly synthesized FGF-2 that becomes coupled to the binding protein within the cell. In the latter case, it must be questioned where within the cells the FGF-2 and binding protein may interact. FGF-2 lacks a conventional signal peptide within the precursor form and is not posttranslationally modified and secreted by the conventional route of the rough endoplasmic reticulum and Golgi apparatus (41). Rather, a distinct vesicular intracellular transport system has been proposed (42) that might allow association with intracellular binding proteins. Exogenous FGF-2 of 18 kDa is capable of being internalized by vascular endothelial cells *in vitro* and subsequently transported to the nucleus (4). However, several cell types also express endogenous forms of FGF-2 with mol masses of 22 and 24 kDa resulting from the use of alternate translation initiation sites (18, 43). These amino-terminally extended peptides are preferentially localized to the nucleus in late G_1 of the cell cycle (15, 19). At least two nuclear localization sequences are, therefore, likely to exist in FGF-2 molecules, one within 18-kDa FGF-2 and a second within the amino-terminal extension present in the 22- and 24-kDa species (17). We have previously shown that ovine fetal growth plate chondrocytes synthesize FGF-2 of 18 and 23 kDa, both of which are capable of nuclear translocation (8).

The function of FGF-2 within the nucleus of chondrocytes can only be speculated upon. It has been suggested that FGF-2 may regulate gene transcription directly in a specific manner in a cell-free system (11). FGF-2 was found to enhance transcription of the *pgk-2* gene while inhibiting the *pgk-1* gene. Within vascular endothelial cells, FGF-2 was found to localize preferentially within the nucleolus, to increase RNA polymerase-1 transcriptional activity in isolated endothelial cell nuclei, and to activate a nucleolar protein kinase, casein kinase II, which phosphorylates an abundant nucleolar protein, nucleolin (4, 9, 12). Proteolytic cleavage of nucleolin is known to trigger ribosomal gene transcription *in vitro*. It is not known whether these events relate primarily to the effects of FGF-2 on cell proliferation or to the state and progression of differentiation of the cell type. Nuclear localization of FGF-2 by isolated growth plate chondrocytes was found to be independent of the effects of endogenous FGF-2 as an autocrine mitogen (8). We have shown that growth plate chondrocytes *in situ* express both FGF-2 and FGFR1 messenger RNAs, that a nuclear presence of FGF-2 peptide is frequently seen by immunohistochemistry, and that expression of both FGF-2 and its receptor are lost as chondrocytes undergo differentiation to postmitotic, hypertrophic cells (44). We have also visualized an increased appearance of the 46- to 48-kDa protein species at the nucleus in the presence of mitogenic stimulants, which suggests that the mitogenic state of growth plate chondrocytes influences the presence of this binding protein species. This truncated receptor form may be involved in the nuclear translocation of FGF-2 in an effort to maintain the undifferentiated proliferative phenotype of the cells.

Acknowledgments

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