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Evidence for a Role of the Alternatively Spliced ED-I Sequence of Fibronectin during Ovarian Follicular Development*

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ABSTRACT

This study was aimed at testing the hypothesis that different forms of fibronectin (FN), produced as a consequence of the alternative splicing of the precursor messenger RNA, play specific roles during development of the ovarian follicle. In particular, we were interested in determining the effect of the ED-I (also termed ED-A) type III repeat, which is absent in the plasma form. Analysis of FN levels in follicular fluids corresponding to different stages of development of bovine follicles revealed marked changes in the concentrations of ED-I$^+$FN, whereas total FN levels remained relatively constant. ED-I$^+$FN levels were higher in small follicles, corresponding to the phase of granulosa cell proliferation. The hypothesis of a physiological role for ED-I$^+$FN was further supported by the finding of a regulation of the alternative splicing of FN in primary cultures of bovine granulosa cells by factors known to control ovarian follicular development. cAMP produced a 10-fold decrease in the relative proportion of the ED-I region. In contrast, transforming growth factor-β elicited a 2-fold stimulation of overall FN synthesis and a 4-fold increase in the synthesis of ED-I containing FN. This effect was evident at the protein (Western blots) and messenger RNA (Northern blots) levels. Although a negative correlation ($P < 0.001$) was detected between ED-I$^+$FN and estradiol levels in follicular fluid, this steroid was unable to modulate *in vitro* the alternative splicing of FN. A possible mitogenic effect of ED-I$^+$FN was suggested by the observation that a recombinant peptide corresponding to the ED-I domain stimulated DNA synthesis in a bovine granulosa cell line (BGC-1), whereas a peptide corresponding to the flanking type III sequences had no effect. The hypothesis of ED-I$^+$FN as a growth regulatory factor was further strengthened by the fact that depletion of FN from BGC-1-conditioned medium, which contained ED-I$^+$FN, abrogated its mitogenic activity, whereas plasma FN was without effect. We propose that changes in the primary structure of FN may mediate some of the effects of gonadotropin and intraovarian factors during follicular development. (*Endocrinology* **140:** 2541–2548, 1999)

IT IS NOW widely accepted that during follicular development granulosa cell function is regulated by a complex interplay between gonadotropins and intraovarian factors. Granulosa cells in culture secrete high amounts of fibronectin (FN) (1–3), which is a component of the extracellular matrix (ECM) (4, 5) and one of the major glycoproteins present in follicular fluid (6, 7). Although it has been demonstrated that FN (8) or activation of the FN receptor (3) can induce differentiation of cultured granulosa cells, the role of this protein in follicular development remains elusive.

FN is a dimeric protein composed of similar, but not identical, polypeptides of about 250 kDa, which share a common modular organization. FN exists in different forms, the majority of which arise as a consequence of messenger RNA (mRNA) alternative splicing (Fig. 1). This phenomenon is subjected to tight regulation in both time and space. The ED-I and ED-II exons (also called ED-A and ED-B) are present in the embryonic FNs, whereas in adult life they are practically absent (9, 10). Plasma FN (pFN), which is synthesized in the liver, lacks these exons, which are readily detectable, although in low amounts, in the ECM of some tissues. However, under certain physiopathological circumstances, ED-I containing FN (ED-I$^+$FN) reappears. In general, reexpression of ED-I$^+$FN takes place in proliferating tissues, such as in some tumors (11) or during wound healing (12), suggesting that ED-I$^+$FN may play a role in cell proliferation.

On the other hand, we have previously reported that a bovine granulosa cell line (BGC-1) obtained by spontaneous immortalization, secretes high levels of FN (13). In addition, conditioned medium from this cell line is mitogenic for both BGC-1 cells and primary cultures of bovine granulosa cells (14).

In the present paper, we provide evidence for regulation of the alternative splicing of the ED-I exon of FN during the

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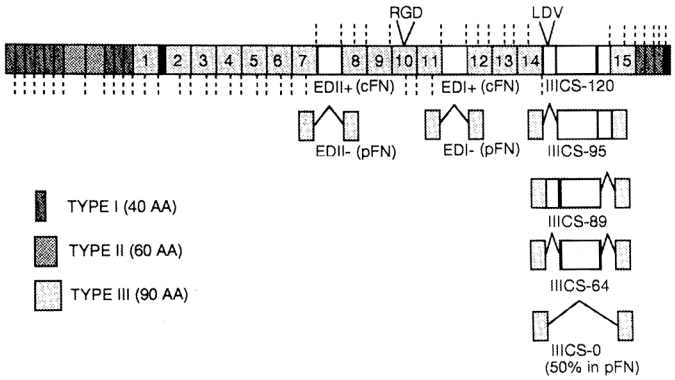

FIG. 1. Variations in the FN primary structure. The *top* represents the longest possible FN polypeptide showing internal homologies. Constitutive (RGD) or alternatively spliced (LDV) cell-binding sites are indicated. *Vertical dashed lines* indicate approximate intron positions determined in the rat gene. Type III repeats are numbered from 1–15. Numbering excludes ED-I and ED-II.

development of the ovarian follicle. This is the first demonstration of such a phenomenon in a normal, nonpathological, process in adult tissues. Moreover, we show data indicating that the ED-I region might exert a growth factor-like activity capable of stimulating granulosa cell proliferation.

Materials and Methods

Reagents and hormones

Recombinant transforming growth factor-β was purchased from R & D Systems, Inc. (Minneapolis, MN); newborn calf serum (NCS) was obtained from Life Technologies (Gaithersburg, MD); human plasma FN was purchased from Boehringer Mannheim (Mannheim, Germany); gelatin-Sepharose 4B was obtained from Pharmacia Biotech (Uppsala, Sweden); radioactive thymidine was purchased from DuPont NEN (Boston, MA; SA, 20 Ci/mmol). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Collagen was prepared from rat tails as previously described (15).

Follicular fluid collection and FN purification

Bovine ovaries were obtained at a nearby slaughterhouse from beef cows and heifers just after slaughter. After transport to the laboratory at 30 C (within 2 h), the ovaries were washed three times with prewarmed saline solution. Follicular fluid was collected by aspiration of follicles of different sizes, and 2 mM phenylmethylsulfonylfluoride was added. Follicular fluid from follicles with diameters between 2–5 mm and between 5–8 mm were pooled in two separate groups; the rest of the follicles (>8 mm) were processed individually. Follicular fluid volume was measured as an indicator of follicle size. Cells were removed by centrifugation at 3,000 $\times$ *g* for 2 min, then each supernatant was cleared by a 10-min centrifugation at 10,000 $\times$ *g*.

FN was purified as follows. Two hundred microliters of each supernatant were diluted in an equal volume of a binding solution (PBS, 0.02% Tween-20, and 2 mM EDTA), and 100 μl of gelatin-Sepharose 4B were added. The sample was kept at room temperature overnight with constant agitation. Low affinity interactions were washed out with 3 M NaCl, and FN was recovered with 8 M urea. Then, the sample was dialyzed against 0.01 $\times$ PBS overnight at 4 C. Finally, each sample was lyophilized, resuspended in 100 μl sample buffer containing β-mercaptoethanol, and boiled for 2 min.

To detect total FN and ED-I$^+$ FN by Western blot, 10 μl of a 1:100 dilution and 10 μl undiluted sample were used, respectively.

RIAs

Progesterone and estradiol levels in follicular fluid were determined by RIA after extraction with ethyl ether as previously described (16).

Establishment of primary cultures

Primary cultures of bovine granulosa cells were established as previously described (14). Briefly, granulosa cells were collected from small follicles (2–8 mm) by aspiration using a needle (18 gauge) and syringe (plastic, 5 ml). Red blood cells were removed by a brief (20-sec) exposure to distilled water. Then, granulosa cells were seeded at 5 $\times$ 10^5 living cells/35-mm culture dish in 2 ml DMEM-Ham's F-12 (1:1) medium supplemented with sodium bicarbonate (2.2 g/liter) in the presence of 10% NCS and antibiotics. Cells were allowed to grow at 37 C in a 5% CO_2 atmosphere for 2 days. At this time cells attained confluence. Cells were washed twice with PBS, and then fresh medium without serum was added with or without the indicated stimuli.

BGC-1 cell culture

BGC-1 cells were obtained by spontaneous immortalization of primary bovine granulosa cell cultures (13). In the present study, we used passage 250 BGC-1 cells. BGC-1 cells were grown on 10-cm diameter plastic dishes in DMEM-Ham's F-12 (1:1) medium supplemented with sodium bicarbonate (2.2 g/liter) in the presence of 5% NCS and antibiotics. For all experiments except those involving DNA synthesis, cells were grown in 5% NCS until confluence was reached. Then, the cells were washed three times with PBS, and serum-free DMEM-Ham's F-12 medium was added with or without the indicated stimuli.

DNA synthesis assay

BGC-1 cells were plated on collagen-coated 96-well dishes at a density of 5 $\times$ 10^3 cells/well in DMEM-Ham's F-12 medium without serum. After 2 h, the indicated stimuli and [^{3}H]thymidine (final concentration, 4 μCi/ml) were added. Twenty-four hours later, cells were harvested with a cell harvester (Nunc A/S, Roskilde, Denmark), and the radioactivity incorporated into the DNA was measured in a liquid scintillation counter.

CCL-64 cells (Mv1Lu, mink lung epithelial cell line, American Type Culture Collection, Manassas, VA) were cultured essentially as previously described (17). Briefly, cultures were maintained in DMEM supplemented with 10% FBS. For thymidine incorporation assays, cells were plated in 96-well plastic dishes at 7.5 $\times$ 10^3 cells/well in DMEM supplemented with 0.2% FBS. After 2 h, the indicated stimuli were added. Twenty-two hours later, [^{3}H]thymidine (final concentration, 10 μCi/ml) was added, and cells were harvested 2 h later. Radioactivity incorporated into the DNA was measured in a liquid scintillation counter.

Recombinant peptides

HisX6-tagged rat recombinant peptides corresponding to the ED-I domain (rrED-I) or to the adjacent domains 11 and 12 (rr11–12) were prepared as previously described (18).

Antibodies

Total FN. Goat antiserum against human FN (Sigma Chemical Co.) was developed using purified human plasma FN and recognizes all FN isoforms.

Anti-ED-I$^+$FN. Mouse monoclonal antibody (IgM) against cellular fibronectin (clone FN-3E2, Sigma Chemical Co.) effectively recognizes cellular FN, but not pFN, in Western blots, as shown in Fig. 2a. Also, this antibody specifically recognizes FN containing the ED-I segment by Western blot (19), as confirmed in Fig. 2b.

Quantitative Western blots

At the end of each experiment, conditioned medium was collected and treated with the protease inhibitor phenylmethylsulfonylfluoride (2 mM). Cell extracts were prepared by the addition of sample buffer containing β-mercaptoethanol and were boiled for 5 min. Both conditioned medium and cell extracts were stored at −20 C until the Western blots were performed.

Gel electrophoresis was performed under reducing conditions in 6% polyacrylamide gels. Total FN and ED-I$^+$ FN accumulated in the supernatant or in the cellular fraction was assessed with standard Western

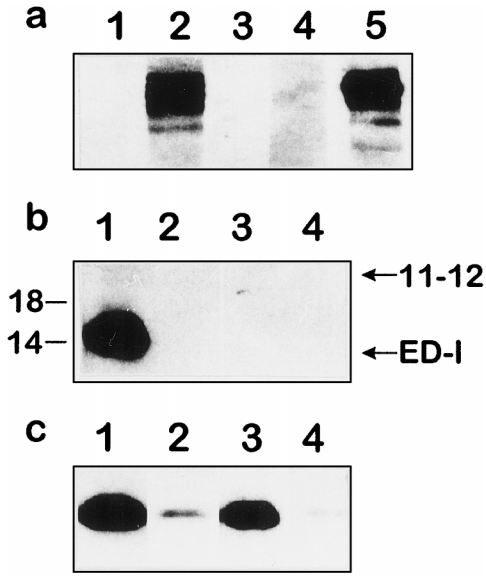

FIG. 2. Western blot showing specificity of the antibodies. a, Anti-ED-I$^+$ FN monoclonal antibody against cellular FN only reacts with cellular FN. Lanes 1–3 were loaded with 1 μg human plasma FN, and lanes 4 and 5 were loaded with a sample containing ED-I$^+$ FN (2 μg protein corresponding to 2-day conditioned medium of TGFβ-treated BGC-1 cells). Lane 1, Normal goat serum; lane 2, antibody against plasma FN; lanes 3 and 5, monoclonal antibody against ED-I$^+$ FN; lane 4, no first antibody (the second antibody was the same one that was used in lanes 3 and 5). b, Anti ED-I$^+$ FN specifically recognizes rrED-I. Lanes 1 and 3 were loaded with 1 μg rrED-I, whereas lanes 2 and 4 were loaded with 1 μg rr11–12. Lanes 1 and 2, Monoclonal antibody against ED-I$^+$FN; lanes 3 and 4, antibody against plasma FN. *Arrows* indicate the mobility of rr11–12 and rrED-I. Molecular mass markers (kilodaltons) are indicated at the *left*. c, Immunoprecipitation of FN present in BGC-1-CM. Aliquots from intact CM from BGC-1 (lanes 1 and 3) and specifically immunoprecipitated CM (lanes 2 and 4) were subjected to PAGE-SDS, as described in *Materials and Methods*. FN was detected using antibody against plasma FN (lanes 1 and 2) and the monoclonal antibody against ED-I$^+$FN (lanes 3 and 4).

blot techniques using the goat antiserum against plasma FN (dilution, 1:1000) or the monoclonal IgM against ED-I (dilution, 1:10000), the appropriate biotin-conjugated second antibody and Extravidin-Peroxidase (Sigma Chemical Co.). Detection was performed with a chemioluminiscence kit (DuPont NEN). For densitometric analysis, an LKB densitometer (Rockville, MD) was used. Under these experimental conditions, the anti-plasma FN antiserum detection threshold is approximately 10 ng, and the anti ED-I antibody is able to detect as little as 0.5 ng recombinant ED-I (data not shown), which corresponds to approximately 11 ng of the 220-kDa FN subunit.

Each Western blot was quantified using a standard curve consisting of serial dilutions of one of the samples included in the assay. Densitometric units were transformed to relative protein concentration units.

Conditioned medium preparation and immunoprecipitation of FN

Conditioned medium (CM) from BGC-1 cells was prepared exactly as previously described (14). FN present in CM was immunoprecipitated essentially as previously described (20). Briefly, goat anti plasma FN (1:100) was added to CM, and the sample was incubated for 90 min at 37 C. Then, rabbit antigoat IgG was added, followed by another incubation of 45 min at 37 C. The immunoprecipitate was collected by a 10-min centrifugation at 10,000 $\times$ *g*. The absence of FN in the supernatant was confirmed by Western blot (Fig. 2). Control immunoprecipitation was performed by replacing the first antibody with normal goat IgG.

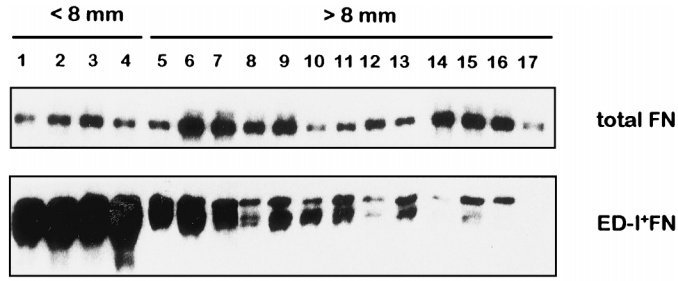

FIG. 3. ED-I$^+$ FN expression in follicular fluid. Western blot for total FN (*upper panel*) and ED-I$^+$ FN (*lower panel*) purified from pools of 2- to 5-mm follicles (lanes 1 and 2; 20 follicles each) or 5- to 8-mm follicles (lanes 3 and 4; 10 follicles each) or from individual follicles bigger than 8 mm in diameter (lanes 5–17). ED-I$^+$ FN was detected in all samples analyzed except sample 17.

RNA extraction and Northern blots

Total RNA from cell cultures was extracted as previously described (21). Transfer of RNA from gels containing formaldehyde to Zeta-Probe membranes and subsequent hybridization were performed following the instructions specified by the manufacturers (Bio-Rad Laboratories, Inc., Hercules, CA). FN mRNA was detected using a 5865-bp *Sal*I/*Hin*dIII probe from a FN mRNA (ED-I$^-$-ED-II$^-$-IIICS89$^+$), and ED-I$^+$ FN mRNA was detected using the M13ED1 probe (160-bp *Pst*I/*Sau*IIIAI fragment of the ED-I exon) (22). Results were normalized using a probe that recognized the glyceraldehyde-3-phosphate dehydrogenase mRNA. Probes used for detection of total FN mRNA and glyceraldehyde-3-phosphate dehydrogenase were labeled using a random priming kit (Life Technologies). The ED-I probe was labeled by primer extension.

Statistical analysis

Western and Northern blots were quantified by densitometric analysis. In the case of the Northern blots, densitometry was validated by direct radioactive counting of the regions corresponding to each band. Data are presented as the mean $\pm$ SEM of measurements from triplicate culture wells. ANOVA was employed to assess the statistical significance of overall treatment effects. Multiple comparisons were performed with Scheffe's test. All experiments were performed at least three times with similar results.

Results

Expression of ED-I$^+$ FN in the ovarian follicle

To determine whether follicular growth was associated with changes in ED-I$^+$ FN, we measured total FN and ED-I$^+$ FN concentrations in follicular fluids collected from follicles at different stages of development. For the initial analysis, follicles were classified in two categories, bigger and smaller than 8 mm in diameter. Figure 3 shows the results obtained in a Western blot from 17 samples of follicular fluid using the anti-pFN antibody, which detects all FN forms (Fig. 3, *upper panel*), and the anticellular FN, which only reacts with the ED-I domain (Fig. 3, *lower panel*). Despite the fact that ED-I$^+$ FN is poorly expressed in adult tissues, we found that not only were 16 of 17 samples positive for ED-I$^+$ FN, but there were important differences in the concentration and relative proportion of ED-I$^+$ FN with respect to total FN among follicles. Lanes 1–4, which correspond to small follicles, displayed higher concentrations and proportions of ED-I$^+$ FN than the rest of the samples.

ED-I-containing FN levels and physiological state of the follicle

To assess the physiological state of each of the follicles analyzed, estradiol and progesterone concentrations were determined. Both the ED-I$^+$ FN concentration (Fig. 4B) and the ED-I$^+$ FN/total FN ratio, which is a measure of the alternative splicing (Fig. 4A), showed an inverse correlation with estradiol levels ($P < 0.002$ and $P < 0.01$, respectively). There was no significant correlation between the total FN concentration and estradiol (Fig. 4C). Neither total FN nor ED-I$^+$ FN levels showed a significant correlation with progesterone concentrations or follicular size (data not shown).

Regulation of ED-I$^+$ FN production in primary cultures of bovine granulosa cells

The data presented above indicated the existence of marked changes in the relative concentrations of ED-I$^+$ FN during follicular development, presumably arising from changes in the alternative splicing of FN mRNA. Therefore, we aimed to identify the hormonal or intraovarian factors capable of regulating this process in primary cultures of bovine granulosa cells. The observation of a strong negative correlation between ED-I$^+$ FN and estradiol levels suggested a possible effect of this steroid. FSH, acting through cAMP, is known to be the main regulatory factor of follicular development and has been shown to modulate FN expression in granulosa cells (13). On the other hand, a putative intraovarian growth factor, transforming growth factor-β (TGFβ) (23), has been previously shown to affect FN mRNA splicing in certain cell lines by stimulating the inclusion of the ED-I exon (24).

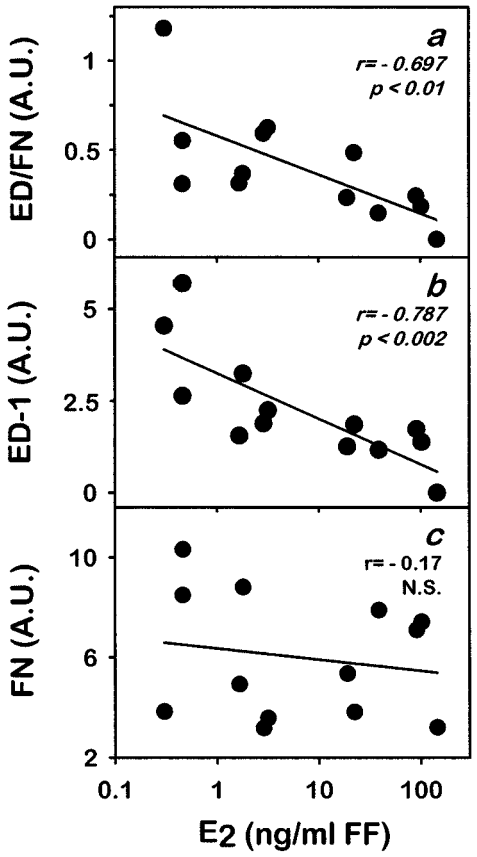

FIG. 4. Correlation between ED-I$^+$ FN, ED-I$^+$ FN/total FN, or total FN and the estradiol concentration in follicular fluids. ED-I$^+$ FN and total FN levels from individual follicles bigger than 8 mm in diameter were quantitated and correlated with the estradiol concentrations in the same samples. Both ED-I$^+$ FN (b) and ED-I$^+$ FN/total FN ratio (a), but not total FN levels (c), showed a significant correlation with the estradiol concentration ($r = -0.787$, $P < 0.002$; $r = -0.697$, $P < 0.01$; and $r = -0.17$; P = NS, respectively).

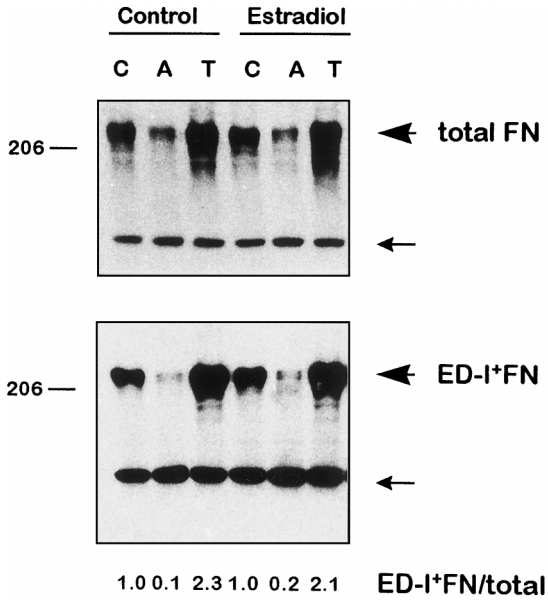

FIG. 5. Regulation of FN and ED-I$^+$ FN expression in primary cultures of bovine granulosa cells. TGFβ increased and cAMP decreased the inclusion of the ED-I domain in the FN molecule; estradiol had no evident effect. Western blot of total FN (*upper panel*) and ED-I$^+$ FN (*lower panel*) from cellular extracts of primary cultures of granulosa cells. Confluent cultures were treated for 48 h with $(Bu)_2$cAMP (A; 1 mM) or TGFβ (T; 5 ng/ml) in the presence (estradiol) or absence (control) of estradiol (100 ng/ml). Control cells (C) did not receive any stimulus. *Large arrows* indicate the position of FN. *Small arrows* show an unrelated polypeptide that reacts with Extravidin. The number at the *left* indicates the position of the 206-kDa molecular mass marker. Numbers at the *bottom* are the relative ratios between ED-I$^+$ FN and total FN (values were normalized against control cultures in the absence of estradiol). Experiments were repeated three times with similar results.

Therefore, cells were treated with either 1 mM $(Bu)_2$cAMP or 5 ng/ml TGFβ for 2 days in the presence or absence of 100 ng/ml of estradiol. The levels of ED-I$^+$ FN and total FN were determined in cellular extracts by Western blots, and the results are shown in Fig. 5. Although $(Bu)_2$cAMP produced a 50% reduction in total FN synthesis and a 20-fold decrease in ED-I$^+$ FN expression (Fig. 5, lanes 1 *vs.* 2), TGFβ caused 2- and 4-fold increases in total and ED-I-containing FN, respectively (Fig. 5, lanes 1 *vs.* 3). Estradiol treatment did not alter either total FN or ED-I$^+$ FN synthesis (Fig. 5, lanes 1, 2, and 3 *vs.* lanes 4, 5, and 6, respectively).

Regulation of FN mRNA alternative splicing in BGC-1 cells

Based on our previous studies showing that the BGC-1 cell line constitutes a valid model for the regulation of granulosa cell function in immature follicles (14), we conducted the rest

of the studies using BGC-1 cells. We determined the effect of TGFβ on FN secretion (Fig. 6a). TGFβ caused a 100% increase in the ED-I$^+$ FN/total FN ratio, results comparable to those obtained in primary cultures (see above).

Levels of mRNA for FN and ED-I$^+$ FN were determined in BGC-1 cultures by Northern blot analysis. As shown in Fig. 6b, treatment with TGFβ increased the mRNA for FN 3-fold and increased that for ED-I$^+$ FN 6-fold.

The ED-I domain stimulates proliferation of granulosa cells

To study a possible function of ED-I$^+$ FN as growth regulator, DNA synthesis experiments were performed using a recombinant peptide comprising the rat ED-I domain (rrED-I) as a stimulus. Figure 7a shows that the addition of rrED-I to the medium stimulated thymidine incorporation in BGC-1 cell cultures in a dose-dependent manner. An equimolar amount of the control rr11–12 peptide (Fig. 7a) did not have any evident effect. Human plasma FN (hpFN), which lacks the ED-I domain, did not show any stimulatory effect even at the highest concentration tested, a concentration that, on a molar basis, is 10 times greater than the lowest effective concentration of ED-I.

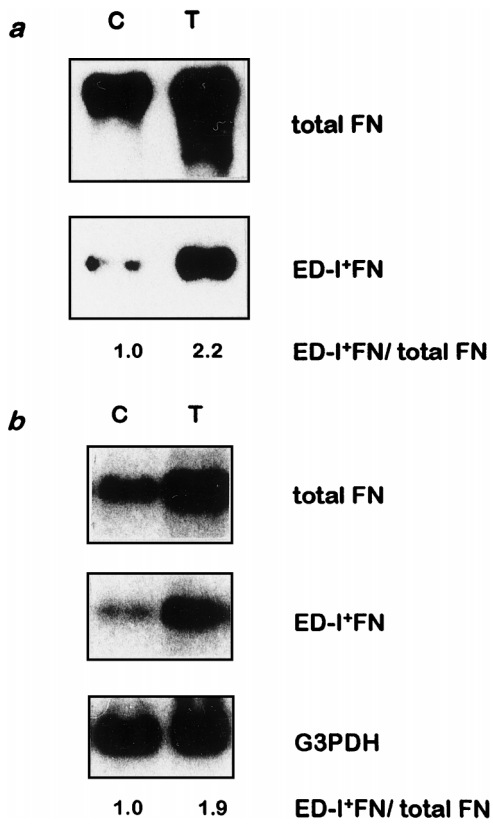

FIG. 6. Regulation of FN and ED-I$^+$ FN expression in the bovine granulosa cell line BGC-1. TGFβ modulates FN mRNA alternative splicing, increasing the inclusion of the ED-I exon (b), an effect that is also evident at the protein level (a). a, Western blot of total FN (*upper panel*) and ED-I$^+$ FN (*lower panel*) from conditioned medium of BGC-1 cells. Cultures were treated for 48 h in the absence (C) or presence of TGFβ (T; 5 ng/ml). Supernatants were collected, and samples were subjected to electrophoresis in 6% polyacrylamide gels under reducing conditions. b, Northern blot of total FN (*upper panel*), ED-I$^+$ FN (*center panel*), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; *lower panel*) mRNA extracted from BGC-1 cells. Confluent cultures were treated for 48 h in the absence (C) or presence of 5 ng/ml TGFβ (T). RNA was extracted, and samples were subjected to electrophoresis in 1% agarose gels under denaturing conditions. Numbers at the *bottom* of a and b are the ratios between ED-I$^+$ FN and total FN (values were normalized against control cultures). Experiments were repeated three times with similar results.

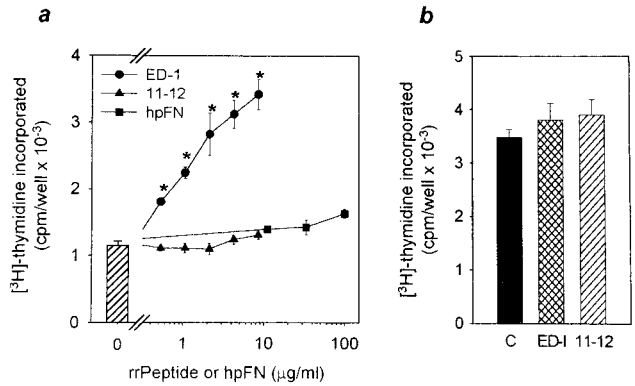

FIG. 7. Regulation of DNA synthesis by rrED-I peptide. a, rrED-I stimulates DNA synthesis in BGC-1 cells. Granulosa cells were seeded, and after 2 h, increasing concentrations of a recombinant peptide containing the ED-I region rrED-I (●), a control recombinant peptide containing the adjacent 11–12 domains rr11–12 (▲), or human plasma FN hpFN (■) were added along with 4 μCi/ml [^{3}H]thymidine. Twenty-four hours later, cells were harvested, and radioactivity incorporated into the DNA was measured. *Asterisks* indicate significant differences ($P < 0.05$) with respect to cultures incubated without stimulus. b, rrED-I does not stimulate DNA synthesis in CCL-64 mink lung cells. CCL-64 cells were cultured in control medium (C), with 10 μg/ml rrED-I (ED-I) or 20 μg/ml rr11–12 (11, 12). [^{3}H]Thymidine incorporation into DNA was performed for 2 h, starting 22 h after plating. Values are the mean ± SEM of triplicate cultures. Experiments were repeated three times with similar results. Results correspond to a representative experiment.

The effect seems to be cell type specific, as other cell types assayed were not susceptible to the ED-I growth-promoting activity. In fact, 1 μM rrED-I failed to stimulate DNA synthesis in Swiss 3T3 fibroblasts and CCL-64 epithelial cells (data not shown and Fig. 7b, respectively). Although CCL-64 cells were cultured in the presence of 0.2% FBS, the same results were obtained in parallel experiments performed in the same culture conditions used for BGC-1 cells (data not shown).

FN synthesized by BGC-1 cells stimulates DNA synthesis

Our data show that the ED-I domain stimulates DNA synthesis in BGC-1 cells and that granulosa cells synthesize and secrete ED-I$^+$ FN. In addition, we have previously demonstrated that BGC-1-CM is mitogenic for BGC-1 cells (14). Taken together, these data suggested that FN present in BGC-1-CM might be one of the molecules responsible for this effect. To address this possibility, BGC-1-CM was prepared, and FN was removed by immunoprecipitation. Figure 2c shows a substantial reduction in both total FN and ED-I$^+$ FN after immunoprecipitation. Then, FN-depleted BGC-1-CM was used as a stimulus in DNA synthesis experiments. As shown in Fig. 8, whereas control CM, immunoprecipitated with normal goat IgG, retained its mitogenic activity, CM did not exhibit any stimulatory effect after FN depletion.

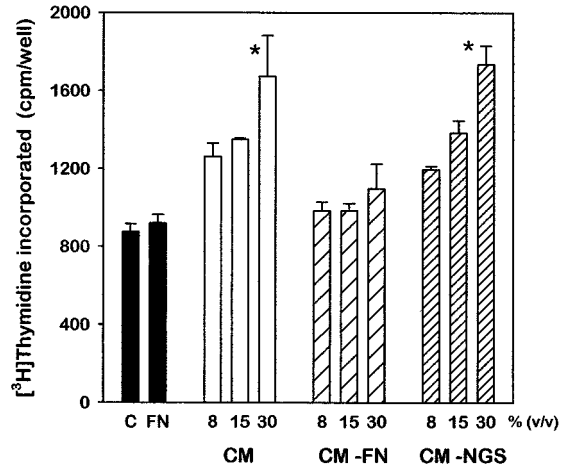

FIG. 8. Immunoprecipitation of FN from BGC-1-CM. FN-depleted CM lacks mitogenic activity. Granulosa cells were cultured with increasing concentrations of BGC-1-CM (CM; *open square*), FN-depleted CM (CM-FN; *wide striped box*), or control immunoprecipitated CM (CM-NGS; *narrow striped box*). As controls, some wells were left untreated (C) or were stimulated with 100 μg/ml hpFN (FN). [^{3}H]Thymidine incorporation into DNA was performed for 24 h. *Asterisks* indicate significant differences ($P < 0.05$) compared with control cultures.

Discussion

Results presented herein demonstrate that there are significant variations in the expression of the ED-I variant of FN during ovarian follicular development. To our knowledge, this is the first report showing significant changes in ED-I$^+$ FN expression during the course of a nonpathological process in the adult. Moreover, our data suggest that ED-I$^+$ FN may play a physiological role, acting as a growth regulator for granulosa cells. We also show that in addition to TGFβ, cAMP can alter the alternative splicing of FN mRNA, thus modulating the production of ED-I$^+$FN within the ovarian follicle.

The ED-I domain during follicular development

The hypothesis that ED-I$^+$ FN plays a physiological role in follicular development rests both on *in vivo* and *in vitro* data. The inverse correlation between intrafollicular levels of ED-I$^+$ FN and estradiol concentrations suggests that ED-I domain expression is under hormonal control.

Huet *et al.* (25) have studied total and ED-I$^+$ FN deposition in thecal and granulosa cell layers by immunocytochemistry of ovine follicles. These researchers found that ED-I$^+$ FN immunoreactivity in thecal cell layers was increased in small and large healthy follicles and decreased in large atretic follicles. Total FN immunoreactivity in granulosa cells, however, reached maximal levels in small atretic follicles. Although direct comparisons cannot be made because these researchers have not evaluated ED-I$^+$ FN levels in follicular fluid, these results would also be consistent with regulation of the alternative splicing of FN during follicular development in the sheep.

Although a contribution of thecal cell-derived FN to follicular fluid cannot be ruled out, the data presented herein, showing the hormonally regulated expression and splicing of FN precursor in granulosa cells, strongly suggest that this latter cell type is mainly responsible for the changes observed in the bovine antrum.

Data concerning the effects of different stimuli on the alternative splicing *in vitro* using both primary cultures and BGC-1 cells offer some clues on the mechanism that would be operating to regulate the *in vivo* concentration of ED-I$^+$ FN. In small follicles that show active proliferation and low response to FSH, TGFβ or a TGFβ-like molecule would be active, stimulating the expression of the ED-I variant of FN. As the follicle matures and acquires the ability to respond to FSH, its second messenger, cAMP, would increase, thus inhibiting ED-I$^+$ FN synthesis and independently increasing estradiol production (26). This would explain the inverse relationship observed between ED-I$^+$ FN and estradiol.

Regulation of FN expression and alternative splicing TGFβ

TGFβ induces FN gene expression in many cell types (27–31), and some of the biological actions of TGFβ may be explained by its effects on FN gene expression. This is exemplified by the rescue of the inflammatory phenotype in TGFβ null mice by the injection of FN synthetic peptides (32). It has been demonstrated that both granulosa and thecal cells are able to produce TGFβs (23). In addition, *in vitro* experiments have shown that this growth factor can regulate granulosa cell growth and differentiation (23). This has led to the hypothesis that members of this family of molecules may be important autocrine/paracrine regulators of follicular development.

Regulation of FN mRNA by TGFβ has not been explored previously in granulosa cells. The increase in FN mRNA by TGFβ treatment reported in the present paper probably reflects induction of the FN gene. Previous reports showed that TGFβ was able to increase the proportion of ED-I$^+$ FN mRNA in cultured cells (33–37). The magnitude of the stimulation achieved with TGFβ treatment is variable and seems to be related at least in part to the basal inclusion of ED-I exon in FN mRNA. Borsi *et al.* (36) reported a 150% increase in cultured fibroblasts (from 10% to 25% of total FN mRNA), whereas Zhang *et al.* (34) showed a 40% stimulation in primary cultures of canine chondrocytes, increasing the proportion of ED-I$^+$ FN from 25% to 35%. Magnuson *et al.* (33) demonstrated that TGFβ increased ED-I inclusion into the FN mRNA in MG65 osteosarcoma and IMR-90 fibroblastic cell lines. The magnitude of the stimulation reported is almost 20% in both cases (from 55–65% of the total FN mRNA). The higher the basal expression of ED-I$^+$ FN mRNA, the lower the stimulation achieved. We extended these findings to granulosa cells showing a 100% stimulation of ED-I inclusion in mature FN RNA after TGFβ treatment.

In addition to the effect on FN mRNA alternative splicing, we were able to show for the first time that this increase is reflected at the protein level. A previous report by Wang *et al.* (38) showed that TGFβ induces polarized secretion of FN in airway epithelial cells. Although they showed that ED-I$^+$ FN was expressed and preferentially secreted through the apical surface only after apical exposure to TGFβ, there was no conclusive evidence of an effect on FN mRNA alternative splicing.

If TGFβ plays indeed a role regulating FN alternative splicing in follicular development, the concentration of its active form should be high in small follicles and decline as the follicle acquires FSH responsiveness and increases estradiol synthesis. In this regard, recent evidence indicates that the production of TGFβ is inhibited by gonadotropins and estradiol in thecal cells (39), whereas granulosa cell TGFβ expression is inhibited by gonadotropin stimulation (40).

cAMP

We have previously reported that FSH inhibits FN synthesis in bovine granulosa cells through a cAMP-mediated process (13). In the present paper we extend those results and show that cAMP is a negative regulator of ED-I exon inclusion in the FN mRNA. To our knowledge this is the first evidence of an involvement of the cAMP signaling pathway in control of the mechanism of alternative splicing. cAMP-dependent phosphorylations could affect the splicing machinery in a direct way or through the induction of relevant genes that, in turn, would regulate FN mRNA alternative splicing.

At present it is unclear whether the levels of cAMP affecting the alternative splicing of FN are compatible with those achieved during the gonadotropin surge, inducing luteinization, or with those required to maintain follicular-type functions of bovine granulosa cells.

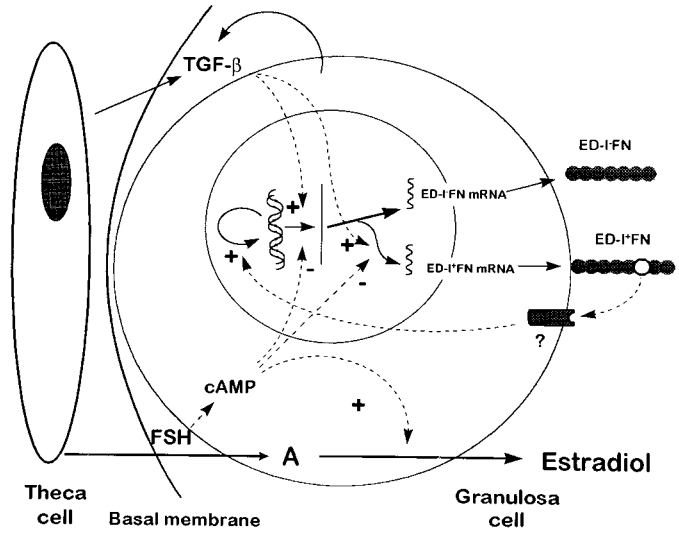

FIG. 9. Regulation of FN expression and action in the bovine follicle. TGFβ, expressed by both granulosa and thecal cells, induces FN gene expression and stimulates inclusion of the ED-I exon. FSH, through its second messenger cAMP, represses the FN gene, inhibits the inclusion of the ED-I exon, and independently stimulates the metabolism of thecal androgens (A) to estradiol. In small follicles, which are low responders to FSH, the concentration of ED-I$^+$ FN is high. This FN variant, through a putative receptor, would stimulate granulosa cell DNA synthesis.

ED-I function

Little is known about the function of the ED-I region. To date, the strongest evidence supporting an *in vivo* role for this domain is that provided by Jarnagin *et al.* (18), who showed that the ED-I region of FN is responsible for the induction of lipocyte differentiation into myofibroblasts in an experimental model of fibrosis induced by bile duct ligation.

Although FN receptors in granulosa cells have not been characterized, our data on the mitogenic action of the ED-I synthetic peptide suggest the existence of specific receptors for this FN domain on the cell membrane. This putative receptor should be different from those reported for FN (41), because hpFN, which lacks the ED-I region, was unable to elicit a similar stimulatory action.

The data presented herein suggest that ED-I$^+$ FN possesses a mitogenic activity on granulosa cells. As the growth-promoting activity of the ED-I domain was determined with a recombinant peptide, further studies are required to verify that this domain has the same activity in the full molecule. However, the hypothesis of ED-I$^+$ FN as a growth regulatory factor is further strengthened by the fact that depletion of FN from conditioned medium containing ED-I$^+$ FN abrogates its mitogenic activity, whereas plasma FN was without effect. Stimulation of DNA synthesis by ED-I seems to be cell type specific, as Swiss 3T3 fibroblasts and CCL-64 mink lung epithelial cells were not responsive to rrED-I.

Another possible mechanism for the growth-promoting action of ED-I$^+$ FN may be sensitization to mitogenic stimulation by an autocrine/paracrine factor. In this regard it has been shown that FN enhances Leydig cell proliferation stimulated by a Sertoli cell-secreted factor (42).

It has been speculated that ED-I$^+$ FN may display differential biological actions due to the marked changes in the spatial structure associated with the introduction of the ED-I exon (43). However, our data showing that the recombinant peptide corresponding to the ED-I exon produces a significant effect would support the idea of an independent binding site for this domain in the cell surface.

The possibility that the ED-I domain conferred growth factor activity to the FN molecule sheds new light on the function of FN within the ovarian follicle. In small follicles, where ED-I$^+$ FN concentration is high, this FN variant may contribute to the process of granulosa cell proliferation.

In larger follicles, where the process of follicular selection is taking place, we found a negative correlation between ED-I$^+$ FN and estradiol levels. As discussed above, this later observation would be consistent with the increased levels of this FN isoform in the ECM of atretic ovine follicles reported by Huet *et al.* (25). However, direct comparisons cannot be made because the soluble ED-I$^+$ FN present in the follicular fluid may elicit different effects than those produced by that deposited in the ECM. In this regard, preliminary data indicate that coating culture dishes with the recombinant ED-I peptide produces a marked inhibition of cell adhesion (data not shown). Furthermore, it has been recently shown that the soluble recombinant domain ED-I can inhibit the effect of ED-I-containing polymerized FN in the induction of the myofibroblastic phenotype (44). Further studies will be required to establish a causal relationship between levels of ED-I$^+$ FN in follicular fluid and the fate of an individual follicle.

Finally, the data presented herein suggest a novel form of regulation (Fig. 9) by which changes in the primary structure of FN may mediate some of the effects of gonadotropin and intraovarian factors during follicular development.

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