Chapter 4

Purification of a candidate gonadotropin surge-inhibiting/attenuating factor (GnSIF/AF) showing MAPK as a possible target

Marja-Liisa Hendriks
Cornelis B. Lambalk
Marco N. Helder
Jurrien de Koning

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ABSTRACT

Gonadotropin surge-inhibiting/attenuating factor (GnSIF/AF) has been known for over two decades, but its molecular structure has not been completely characterized yet. In the last 20 years, five different putative GnSIF/AF sequences have been published. In this article, we describe a procedure to isolate and characterize GnSIF/AF from bovine follicular fluid. A GnSIF/AF-derived synthetic peptide (SP-GnSIF/AF) was produced, and the intracellular bioactivity of GnSIF/AF was tested for intracellular action with a MAPK-assay. Two different bioactive molecular weight forms of GnSIF/AF were isolated, a 160-kDa heteromeric and a monomeric 40-kDa protein. The 40-kDa form appeared to be a subunit of the 160-kDa protein. The synthetic peptide mimicked the actions of GnSIF/AF, such as inhibition of GnRH-induced LH secretion and attenuation of the MAPK phosphorylation. The two GnSIF/AF candidates do not show similarities with previously published GnSIF/AF sequences. These are the first data showing the influence of GnSIF/AF on intracellular processes involved in GnRH self-priming and that the biological action of GnSIF/AF was preserved for the produced synthetic peptide. The results provide strong evidence that the identified candidate proteins are the true GnSIF/AF.

INTRODUCTION

Gonadotropin surge-inhibiting factor (GnSIF, also called gonadotropin surge-attenuating factor, GnSAF) has been known for over two decades, yet its molecular structure has still not been completely characterized. GnSIF/AF is an ovarian product, originating from the granulosa cells (Fowler et al., 2002), and its production is stimulated by follicle stimulating hormone (FSH) (Messinis et al., 1991; Fowler and Price, 1997). GnSIF/AF, in turn, inhibits the gonadotropin-releasing hormone (GnRH)-induced pituitary luteinizing hormone (LH) secretion (Messinis and Templeton, 1991; Fowler et al., 2003) and antagonises the self-priming effect of GnRH, both in vivo (Messinis and Templeton, 1991) and in vitro (Koppenaal et al., 1992; Fowler et al., 1994; Byrne et al., 1996; de Koning et al., 2001). GnSIF/AF acts in a functional antagonistic way with GnRH to regulate LH levels, and this seems crucial for normal folliculogenesis and consequently regular menstrual cycles (de Koning et al., 2001; Fowler et al., 2003; Messinis, 2006). Dysfunction in the GnSIF/AF synthesis or action could play a role in cycle disturbances associated with elevated LH levels, like polycystic ovarian syndrome (Hendriks et al., 2008) and low GnSIF/AF bioactivity is found in women with reduced ovarian reserve (Martinez et al., 2002).

Identification of GnSIF/AF

So far, GnSIF/AF has not been fully and convincingly characterized. Multiple attempts to identify GnSIF/AF have been performed and it has become clear that it is a difficult-to-characterize protein. This is partly due to the high concentration of sex steroids in the biological fluids used and the co-elution with albumin and immunoglobulins (Fowler et al., 2003). Low concentrations of bioactive GnSIF/AF remain after purification, making identification a challenge (Fowler et al., 2003). In addition, GnSIF/AF might be a labile protein, and the N-terminal part could be insensitive to the amino acid sequencing procedures used (de Koning et al., 2001).

Five attempts to identify GnSIF/AF have been published and GnSIF/AF has been isolated in serum and follicular fluid of women and pigs, and in Sertoli cell-enriched medium of male rats (Tio et al., 1994; Danforth and Cheng, 1995; Mroueh et al., 1996; Pappa et al., 1999; Fowler et al., 2002). All five attempts have resulted in different amino acid sequences and molecular weights, varying from 12.5 to 69 kDa (Table 1a and b). Three research groups have found a biologically active GnSIF/AF protein of around 64 kDa in porcine follicular fluid and in human granulosa-luteal cell conditioned medium, although the reported NH2-terminal amino acid sequences did not match (Danforth and Cheng, 1995; Mroueh et al., 1996; Fowler et al., 2002). Furthermore, a 37 kDa protein was found by Tio et al. (1994) in Sertoli cells from rats and Pappa et al. (1999) isolated a 12.5 kDa fragment from human follicular fluid. Most publications did not include GnSIF/AF bioactivity linked to protein bands after native polyacrylamide gel electrophoresis

(PAGE) (Tio et al., 1994; Danforth and Cheng, 1995; Mroueh et al., 1996; Pappa et al., 1999), thus the proteins in the purified fractions cannot conclusively be indicated as GnSIF/AF. Only the latest publication by Fowler et al. (2002) showed bioactivity coupled to protein bands after PAGE, and demonstrated that a polyclonal antiserum raised against GnSIF/AF was able to block the GnSIF/AF bioactivity in vitro. Unfortunately, the antiserum was not suitable for further characterization of GnSIF/AF (Fowler et al., 2002).

Pure GnSIF/AF is expected to reduce GnRH-stimulated LH secretion only and not that of FSH (Danforth et al., 1987). The 37 kDa protein found by Tio et al. (1994) caused reduction in both GnRH stimulated LH and basal FSH secretion, suggesting that the protein may not be pure GnSIF/AF, or could represent a male variant of GnSIF/AF with different biological characteristics.

Recently it has been demonstrated that, part of the human serum albumin (hSA) gene is expressed in human granulosa cells, and there is a possible correlation with GnSIF/AF (Karligiotou et al., 2006). One of the amino acid sequences of the putative GnSIF/AF has shown homology to hSA (Pappa et al., 1999) and a recombinant sub domain of hSA has exhibited GnSIF/AF bioactivity (Tavoulari et al., 2004). Furthermore, a GnSIF/AF antibody identified a hSA precursor of the 66 kDa protein (Sorsa-Leslie et al., 2005). However, hSA is not GnSIF/AF, as the full-length protein does not exhibit GnSIF/AF bioactivity (Tavoulari et al., 2004), but GnSIF/AF might be formed by precursor products of this albumin gene by alternative splicing (Karligiotou et al., 2006).

Intracellular Action of GnSIF/AF

Little is known about the intracellular interaction between GnRH and GnSIF/AF in the pituitary gland. GnRH stimulates the pituitary release of LH and FSH directly and indirectly by a self-priming process through induction of de novo protein synthesis, the so-called self-priming associated proteins (SPAP) (Aiyer et al., 1974; de Koning et al., 2001). GnRH acts through second messengers, like cyclic-AMP and diacylglycerol (DAG) (de Koning et al., 2001), and increases the enzyme mitogen-activated protein kinase (MAPK) activity by phosphorylation (Mitchell et al., 1994), a step upstream from the synthesis of SPAP. GnSIF/AF antagonizes this self-priming of the pituitary, probably by preventing the synthesis and/or by neutralizing the actions of SPAP (Tijssen et al., 1997), resulting in a reduction of serum LH levels. This inhibitory effect of GnSIF/AF might work via inactivation of MAPK itself and/or downstream controlled steps. This possibility was tested here.

In this article, we describe a procedure to isolate and characterize GnSIF/AF from bovine follicular fluid (bFF) and produced a GnSIF/AF-derived synthetic peptide (SP-GnSIF/AF). Furthermore, the intracellular interaction betweenGnRHand GnSIF/AF was tested through aMAPKassay, using highly purified GnSIF/AF products and the synthetic peptide derived from these preparations.

Table 1a. Published Purification Attempts of GnSIF/AF

Author	MW (kDa) of main GnSIF/AF protein	MW (kDa) minor GnSIF/AF bioactivity	GnSIF/AF activity proved by	Source	Species
Tio et al. (1994)	37	—	Bioactivity	Sertoli cell-enriched medium	Rat
Danforth and Cheng (1995)	69	29	Bioactivity	Follicular fluid	Pig
Mroueh et al. (1996)	63	40, 46, 52, 59, 77, 90, 104	Bioactivity; GnSIF antibody	Follicular fluid (superovulated women)	Human
Pappa et al. (1999)	12.5	—	Bioactivity	Follicular fluid (superovulated women)	Human
Fowler et al. (2002)	64	17, 83	Bioactivity; polyclonal antibody	Follicular fluid (superovulated women) Granulosa/ luteal cells from spontaneous cycles	Human

Table 1b.

Author	Amino acid sequence
Tio et al. (1994)	NH_2-SDXXPQL
Danforth and Cheng (1995)	NH_2-SLPLAG (69kDa subunit); NH_2-LXPGSSXXTVXX (29kDa subunit)
Mroueh et al. (1996)	—
Pappa et al. (1999)	NH_2-ALEVDETYVPK–XXALVELVK-AVMDDFAAFVEK
Fowler et al. (2002)	Internal: EPQVYVHAP; NH_2-XVPQGNAGN

RESULTS

Purification Steps of GnSIF/AF

Purification of bFF was initiated with stepwise ammonium sulfate precipitation. Total protein content and the relative bioactivities of the sediments of bFF obtained after five steps of cumulative saturation with ammonium sulfate from0% to 100% were analyzed. The 20–40% ASP fraction showed the highest percentage of GnSIF/AF bioactivity (75%) and was used for hydrophobic interaction chromatography (HIC). The protein elution profile and bioassay data of this HIC are shown in Figure 1. GnSIF/AF and inhibin bioactivity was found mainly in fractions 39–74 (inhibition of GnRH-stimulated LH release and basal FSH release by 64%and 61%, respectively). Fractions 61–74 were used for further purification on the fast protein liquid chromatography gel filtration (GFC–FPLC) (Figure 2). GnSIF/AF and inhibin bioactivity was found in fractions 28–32 (pool 1; high molecular weight (HMW)-GnSIF/AF), 34–38 (not subjected to further purification) and 43–48 (pool 2; low molecular weight (LMW)-GnSIF/AF). GnRH-stimulated LH release and basal FSH release in pool 1 were inhibited by 57% and 36%, respectively; in pool 2, the inhibitions were 64% and 53%, respectively. The two pools were independently purified further on an anion exchange chromatography (AEC). The protein elution profile and bioassay data of the HMW-GnSIF/AF fraction (pool 1) and LMW-GnSIF/AF (pool 2) are given in Figures 3 and 4, respectively. The HMW-GnSIF/AF was bound onto the column and was found in fractions 38–42 (inhibition of GnRH-stimulated LH release and basal FSH release by 46% and 35%, respectively), in contrast to the LMW-GnSIF/AF, which was not bound onto the column and was eluted in the run through fractions 3–15 (inhibition of GnRH-stimulated LH release and basal FSH release by 22%and 64%, respectively). Subsequently, the HMW- and LWM-GnSIF/AF fractions underwent GFC–HPLC. Bioactivity of the HMW-GnSIF/AF was found in fractions 11–17 (inhibition of GnRH-stimulated LH release and basal FSH release by 24% and 45%, respectively) and bioactivity of the LMW-GnSIF/AF in fractions 17–22 (inhibition of GnRH-stimulated LH release and basal FSH release by 53% and 50%, respectively).

Further Characterization of the HMW-GnSIF/AF Fraction

After native PAGE of the HMW-GnSIF/AF preparation, four more-or-less dominant protein bands were assayed for GnSIF/AF bioactivity (Figure 5). Bioassay showed that the second band (Figure 5, gel piece 2) was bioactive. Nonreduced sodium dodecyl sulfate (SDS)–PAGE of this fraction showed a molecular weight of 160 kDa (not shown). The protein band in the second gel piece significantly inhibited LH release, thereby displaying a clear GnSIF/AF bioactivity; however, no significant effect on FSH and thus inhibin activity was observed. Reduced SDS–PAGE (Figure 6a,b) of this bioactive protein band showed two bands of 40 kDa and one band of 64 kDa, indicating that HMWGnSIF/AF may consist of multiple subunits (Figure 6a, lane 2).

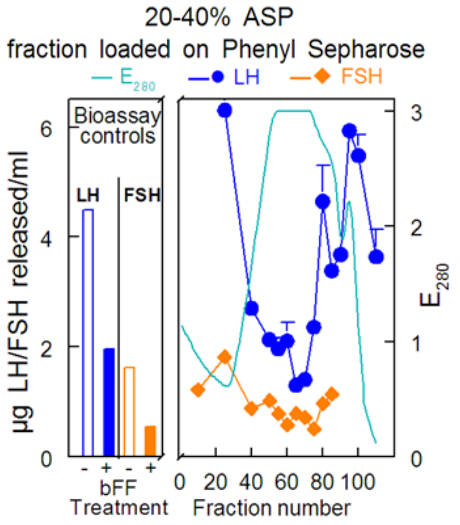

Figure 1. Hydrophobic interaction chromotography (HIC) with a Phenyl Sepharose column, loaded with 20–40% ASP fraction. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions. E280: protein content was measured using E280nm absorption.

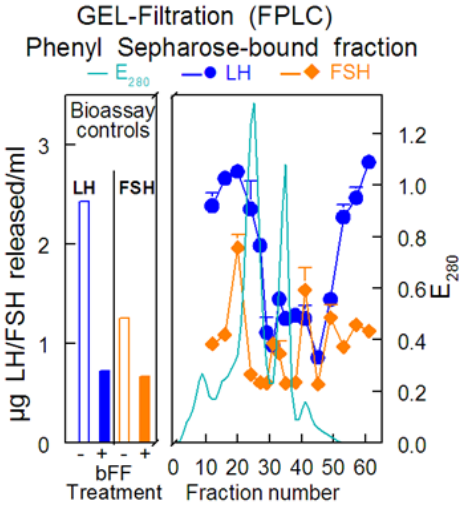

Figure 2. Gel filtration chromatography with a Hiload Superdex FPLC column, loaded with the Phenyl Sepharose-bound fraction. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions. E280: protein content was measured using E280 nm absorption.

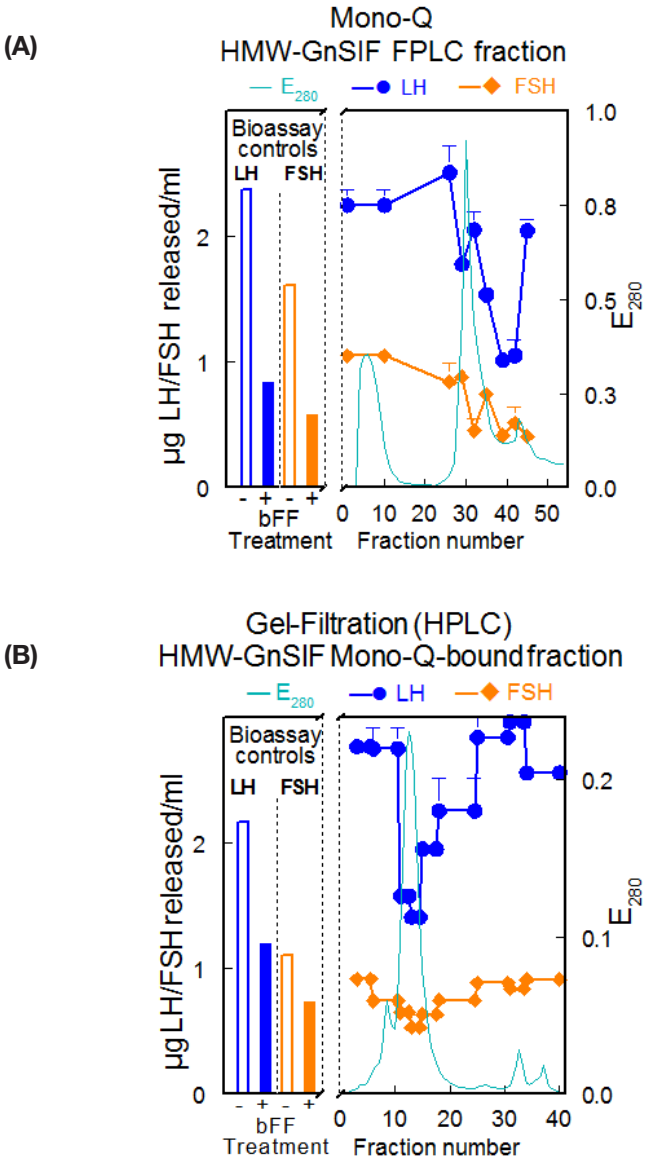

Figure 3. (A) The HMW-GnSIF/AF pool was further purified by anion exchange chromatography on a FPLC mono column. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions.
(B) The bound fraction was subjected to gel filtration chromatography on a HPLC Shodex 800 column. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions. E280: protein content was measured using E280nm absorption.

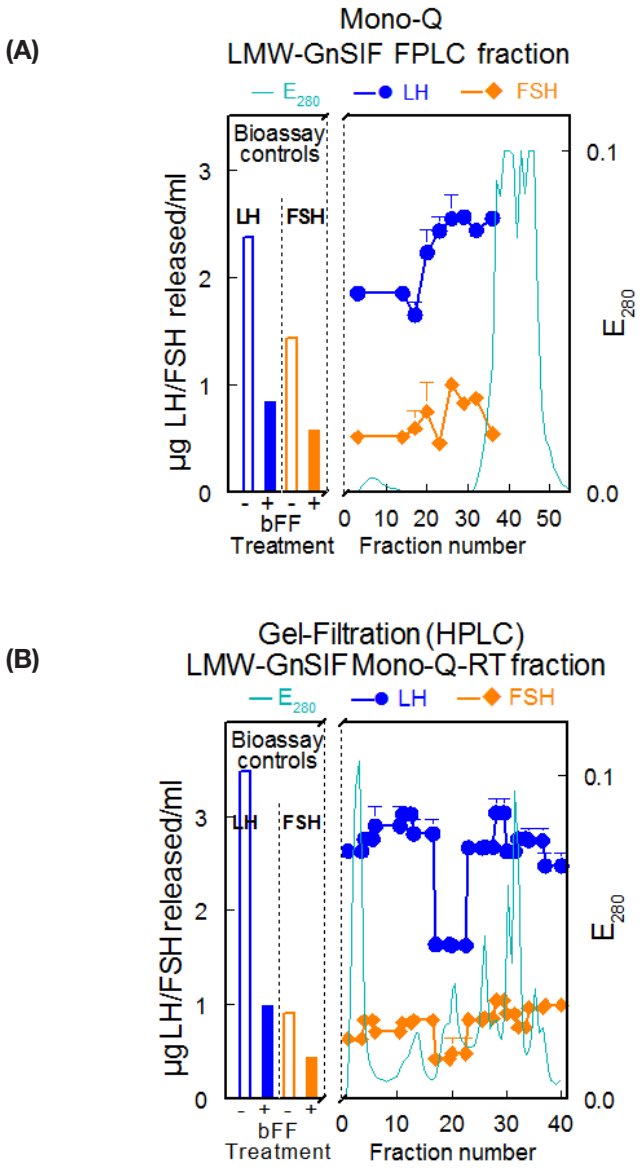

Figure 4. (A) The LMW-GnSIF/AF pool was further purified by anion exchange chromatography on a FPLC mono column. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions.

(B) The non-bound fraction was subjected to gel filtration chromatography on a HPLC Shodex 800 column. The bars on the left indicate the lower and upper limits of the bioassay controls. The lines on the right show the LH and FSH release per ml for various fractions. E280: protein content was measured using E280 nm absorption.

A blot of the HMW-GnSIF/AF preparation after reduction and SDS–PAGE was made, and the three major bands (above indicated as possible GnSIF/AF candidates) were subjected to amino acid sequence analysis. Sixteen amino acid residues were elucidated from the highest molecular weight band of approximately 64 kDa and 26 residues from one of the 40 kDa proteins (Table 2). The other 40 kDa protein (the upper band) appeared to be N-terminally blocked and no amino acid sequence could be recovered. The N-terminal sequences were coupled to the online Genbank to match the proteins with known sequences. The 64 kDa protein showed high homology with human and bovine b-chain compliment C3 (human: identities, 14/15 and positives, 15/15 amino acids; bovine: identities, 16/16 and positives, 16/16 amino acids) and the 40 kDa sequence with the internal fragment of human synovial stimulatory protein (hSSP; identities, 23/25 and positives, 24/25 aminoacids)terminal parts of Fe-hydrogenase Desulfovibrio desulfuricans (FDDD; identities, 16/20 and positives, 18/20 amino acids) and human urinary tract stone matrix protein (hUTSMP; fragment of 40 kDa; identities, 9/11 and positives, 10/11 amino acids) (Table 2).

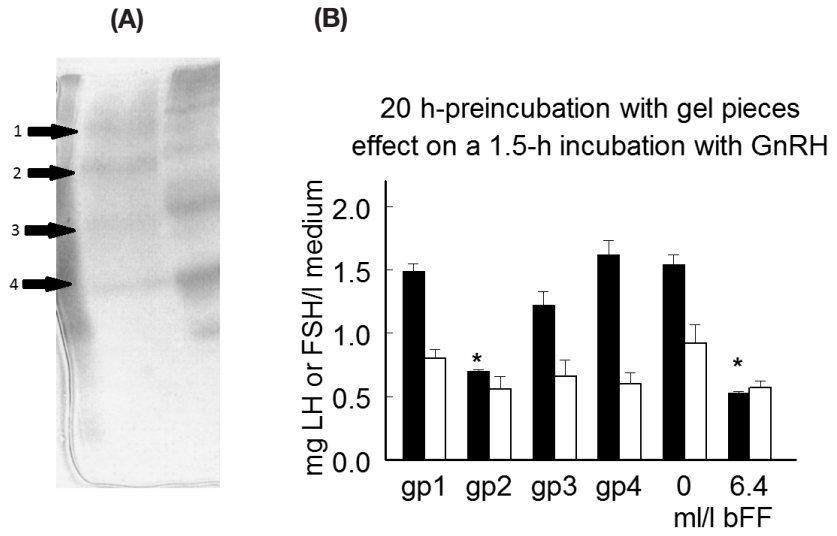

Figure 5. A Showdex-800 HPLC gel filtration purified HMW-GnSIF/AF pool (as in Fig. 3a) was subjected to native gel-electrophoresis.

(A) The bioactivity of GnSIF/AF and inhibin was determined for the 4 most-pronounced protein bands (gel pieces: gp1, gp2, gp3, and gp4)

(B) FSH concentrations (white bar) were measured at the end of a 20 hr pre-incubation. There were no significant differences among the groups (analysis of variance). LH concentrations (black bar) were measured 1.5 hr after change of the medium for fresh medium containing 1 mmol/L GnRH. Results are expressed as mean ±SEM (n=3). LH release was inhibited significantly (in gp2 and bovine follicular fluid (bFF)) compared to all other groups (Analysis of variance, Duncan's multiple comparison test, *P<0.05).

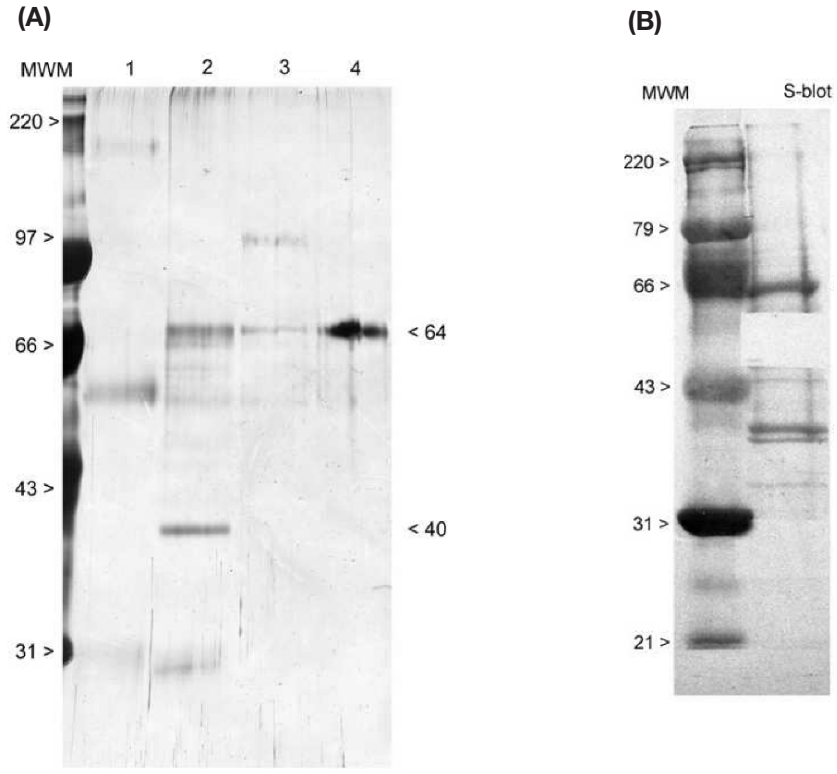

Figure 6. (A) Reduced SDS–PAGE gel. Lanes 1–4: gel pieces obtained after native gel electrophoresis of the HPLC–GF pool of HMW-GnSIF/AF (see Fig. 5). Lane 2 is the most bioactive HMW-GnSIF/AF, existing of two subunits of 64 and 40 kDa.
(B) Sequencing blot of HMW-GnSIF/AF (right lane) obtained after HPLC–GF. Molecular weight markers (MWM) are shown on the left.

Table 2. HMW- and LMW-GnSIF/AF and proteins with high homology

Protein	MW (kDa)	A.a. sequence
HMW GnSIF	64	D/N P M Y S M I T P N I L R L E S
human-ßC3	75	S P M Y S I I T P N I L R L E S
bovine- ßC3	190	D/N P M Y S M I T P N I L R L E S
LMW GnSIF	40	D I N G G G A T L P Q P L Y Q T S G V L T A F G A P
hSSP	205 (3x70)	D I N G G G A T L P Q P L Y Q T A A V L T A F G A
FDDD	54 (42.5 & 11)	D V V P G G A T L P Q P L Y Q T A G V L
hUTSMP	40	G G A T L P E K L Y

Further Characterization of the LMW-GnSIF/AF Fraction

Native PAGE of the LMW-GnSIF/AF pool obtained after HPLC–GF failed, because it did not enter the gel. When this pool was subjected to SDS–PAGE, under both reduced (Figure 7a, lane 1) and non-reduced (not shown) conditions two 40 kDa protein bands were detected (Figure 7a,b). For comparison, in the experiment displayed in Figure 7a (lanes 2–5), similar HMW-GnSIF/AF native gel pieces were applied as in Figure 6a (lanes 1–4). The same protein band patterns are seen in corresponding lanes. Also the similarity in location of the 40 kDa bands in the LMW-GnSIF/AF and HMW-GnSIF/AF fractions is observed. After blotting the lowest 40 kDa band of the LMW-GnSIF/AF fraction, 10 amino acids were elucidated in a sequence analysis and were found to be identical to those of the 40 kDa subunit of HMW-GnSIF/AF. Again the other upper 40 kDa band in this lane was N-terminally blocked.

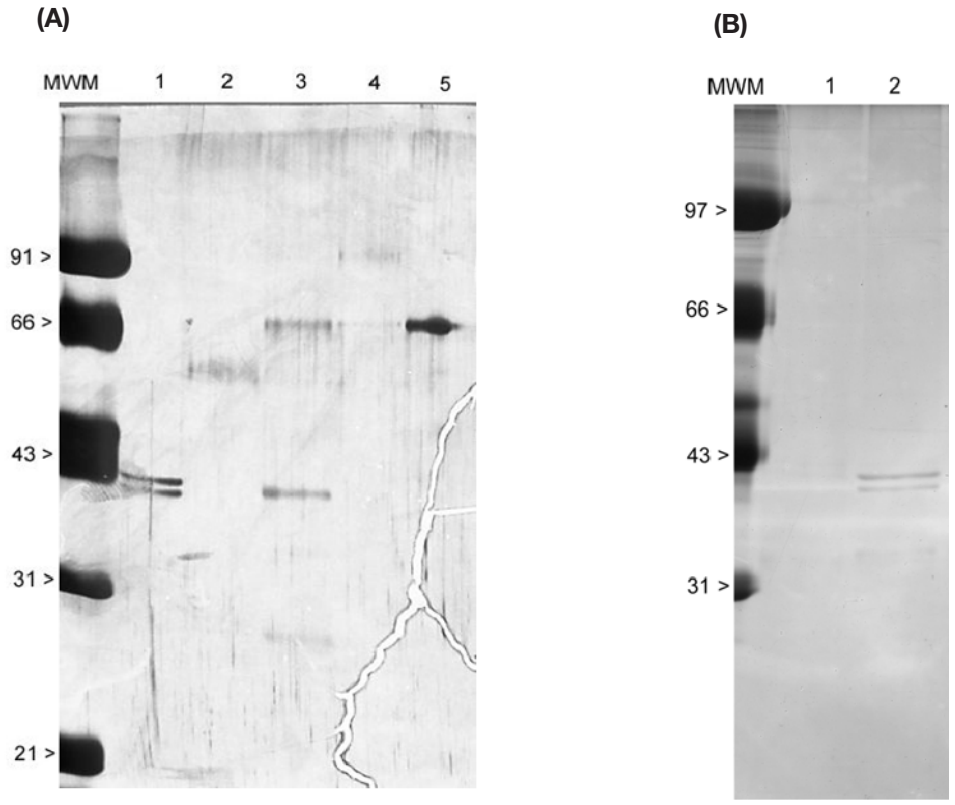

Figure 7. (A) Showdex-800 HPLC gelfiltration purified GnSIF/AF fractions (as in Figs. 3a and 4a) were subjected to reduced SDS–PAGE electrophoresis. Lane 1: LMW-GnSIF/AF fraction. Lanes 2–5: Similar HMW-GnSIF/AF fractions as used in Figure 6a. Molecular weight markers (MWM) are shown on the left.
(B) Sequencing blot of LMW-GnSIF/AF (lane 2) obtained after HPLC–GF. MWM are shown on the left.

SP-GnSIF/AF

The N-terminal sequence of the common 40 kDa GnSIF/AF subunitwas used for the production of a synthetic 26 amino acid GnSIF/AF peptide (SP-GnSIF/AF). The SP-GnSIF/AF had a significant inhibitory effect in three separate bioassays on the GnRH stimulated LH release ($P<0.05$). Basal FSH secretion was not affected by SP-GnSIF/AF (Figure 8).

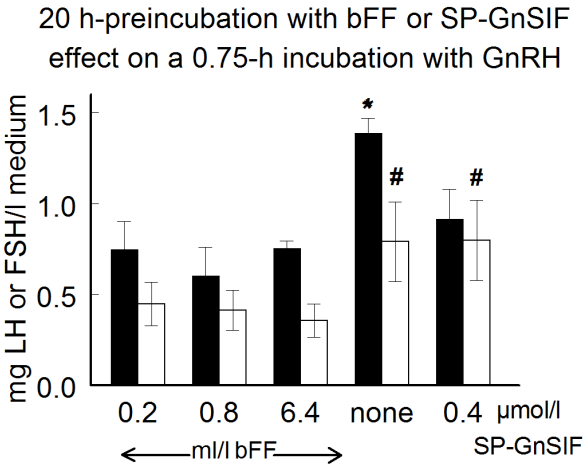

Figure 8. Effect of a 20 hr incubation of primary pituitary cells from immature female rats with or without bovine follicular fluid (bFF) or synthetic peptide (SP)-GnSIF/AF on basal FSH (white bar) and LH release (black bar). LH was measured after a subsequent 45-min period (within the LH lag-phase response) with fresh medium to which GnRH (1 mmol/L) was added. #:$P<0.05$, compared with all bFF-treated groups. *:$P<0.05$, compared with all other groups (Analysis of variance/Duncan's multiple comparison test).

MAPK Results

GnRH increased the intracellular MAPK activity by 60–100% in primary pituitary cells and about 200% in αT3-1 cells above control values (Figure 9a–c). All the GnSIF/AF bioactive preparations (LMW-, HMW-GnSIF/AF, and SP-GnSIF/AF) significantly decreased GnRH-stimulated MAPK activation in primary pituitary cells ($P<0.05$). The effect was strongest when GnSIF/AF or SP-GnSIF/AF was added 10 min after the start of exposure of the cells to GnRH, thus requiring only 5 min of co-incubation. Addition of the GnSIF/AF preparations without GnRH did not significantly affect the MAPK-P/MAPK ratio. MAPK activity in αT3-1 cells was not affected by the GnSIF/AF preparations alone or in combination with GnRH (results not shown).

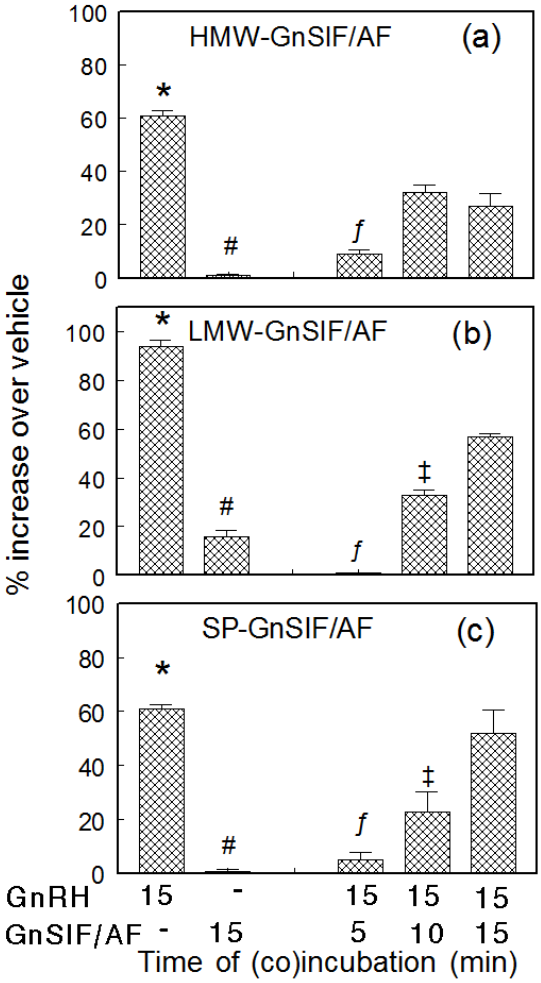

Figure 9. The effect of various GnSIF/AF bioactivities on the GnRH-induced MAPK phosphorylation with different co-incubation times, varying from 0 to 15 min in primary pituitary cells from immature female rats.
(A) Effect of HMW-GnSIF/AF. *: GnRH-only group versus all other groups, #: HMW-GnSIF/AF-only group versus 10 and 15 min co-incubation groups, and *f*: 5 min co-incubation group versus 10 and 15 min co-incubation groups ($P<0.05$, analysis of variance/ Duncan's multiple comparison test).
(B) Effect of LMW-GnSIF/AF. *: GnRH-only group versus all other groups, #: LMW-GnSIF/AF-only group versus all co-incubation groups, f: 5 min co-incubation group versus 10 and 15 min co-incubation groups, and ‡: 10 min co-incubation group versus 5 and 15 min co-incubation groups ($P<0.05$, analysis of variance/ Duncan's multiple comparison test).
(C) Effect of synthetic peptide (SP)-GnSIF/AF. *: GnRH-only group versus all other groups (except the 15 min co-incubation group), #: SP-GnSIF/AF-only group versus all co-incubation groups, *f*: 5 min co-incubation group versus 15 min co-incubation group, and ‡: 10 min co-incubation group versus 15 min co-incubation group ($P<0.05$, analysis of variance/Duncan's multiple comparison test).

DISCUSSION

Putative GnSIF/AF Proteins

The biological function of GnSIF/AF is well known, but its molecular structure is not conclusively identified. All previous purification attempts, including the present one, have failed to elucidate the full structure of GnSIF/AF (Tio et al., 1994; Danforth and Cheng, 1995; Mroueh et al., 1996; Pappa et al., 1999; Fowler et al., 2002). In this experiment, two proteins (40 and 160 kDa) have been isolated that show GnSIF/AF bioactivity and influence intracellular processes in pituitary cells. The LMW-GnSIF/AF (40 kDa) appeared to be a monomeric protein, contrary to the heteromeric HMW-GnSIF/AF (160 kDa) protein, consisting of three subunits (two units of 40 kDa and one 64 kDa subunit). The first 10 N-terminal amino acids were identical in the HMW and the lower LMW 40 kDa units, and based on this finding the LMW-GnSIF/AF is probably a subunit of the HMW form. Both the LMW- and HMW-GnSIF/AF proteins suppressed the GnRH-stimulated LH secretion, probably by influencing intracellular processes involved in pituitary priming (see below). Furthermore, a SP-GnSIF/AF was produced from the N-terminal amino acid sequence of the common 40 kDa GnSIF/AF subunit and this synthetic peptide displayed GnSIF/AF activity and did not harbor inhibin activity. These results provide strong evidence that the discovered candidate proteins are true GnSIF/AF.

The question rises if the found GnSIF/AF proteins in this study are similar to the known candidate proteins. The molecular weight of approximately 64 kDa has been found previously in humans by Fowler and Mroueh (Mroueh et al., 1996; Fowler et al., 2002). But considering the published amino acid sequences and other biochemical characteristics, they are not likely to be similar proteins. A 40 kDa fraction has also been detected previously, but this protein only displayed minor GnSIF/AF bioactivity and was not bound by their GnSIF/AF antibody (Mroueh et al., 1996). The 40 kDa proteins are difficult to compare, as no amino acid sequence was published by Mroueh et al. (1996). Thus, there is a possibility that the protein found by Mroueh is (partly) identical to ours. The reason for the existence of multiple and putative GnSIF/AF proteins is unclear, but may be due to the use of material from different species. Furthermore, the purification procedures used so far have several weaknesses. The choice of bioassays, for example, can cause false positive results, as other accompanying structures in the partly purified fractions may attenuate the GnRH-stimulated LH secretion (de Koning et al., 2001). Every component with a direct or indirect non-specific inhibitory action on LH release might be indicated as a GnSIF/AF candidate. However, since the GnSIF/AF bioactive preparations did not affect MAPK activity in the αT3 cell line, these results suggest that no such non-specific inhibitory action takes place. In addition, it is possible that multiple proteins are involved in the attenuation of the pituitary LH secretion or that the proteins undergo post-transcriptional modification, as various found proteins have demonstrated GnSIF/AF activity (Pappa et al., 1999; Fowler et al., 2002,

2003). Which of the known GnSIF/AF candidates is the “true” GnSIF/AF remains the question and further experiments are necessary for a definite characterization of GnSIF/AF.

Biological Function and Intracellular Influence of GnSIF/AF

Multiple factiors are involved in LH regulation, the most important regulators are GnRH, progesterone, estrogen, and GnSIF/AF. Progesterone suppresses LH secretion mainly by inhibiting GnRH pulses at the level of the hypothalamus. The suppressive effects are the most obvious in the luteal phase and the beginning of the follicular phase (Anttila et al., 1992).

It is well accepted that, estrogens have a major impact on the release of LH during the cycle. This control consists of negative and positive feedback both at the central level (release of GnRH) and pituitary level (LH response to GnRH). At the same time, estradiol decreases GnRH release and increases pituitary LH responsiveness to GnRH. The resulting levels of LH remain low because of the dominating effect of the inhibition of GnRH release. This is the situation that anticipates the LH surge. It is not unreasonable to assume that during this period, for whatever reason, small increase in GnRH release might cause premature increase in LH levels before the onset of the LH surge. GnSIF/AF is thought to counteract the stimulatory effect of estrogen at the pituitary level by functioning in an antagonistic way with GnRH (de Koning, 1995; Tijssen et al., 1997; de Koning et al., 2001; Dafopoulos et al., 2004). It is the above-described interaction between GnRH and GnSIF/AF that may allow estradiol to control LH levels effectively, and that may prevent unwanted effects of changes in GnRH release and consequently premature luteinization. Their mutual target is the GnRH-dependent process of de novo protein synthesis (the so-called SPAP) that expresses the self-priming phenomenon: the GnRH induced increase in the pituitary LH responsiveness to its own action. Through antagonistic interaction, GnRH and GnSIF/AF control the activity of the involved proteins or subsequently induced steps in the release process (increased by GnRH and decreased by GnSIF/AF), which constitute the rate-limiting step in LH release stimulated by GnRH via the releasing pathway. The protein synthesis dependent action of GnRH to antagonize the effect of GnSIF/AF is a relatively slow process: an increased rate of LH release (or self-priming) is achieved only after some hours. During most of the ovarian cycle, GnSIF/AF neutralizes the priming action of each GnRH pulse and thereby restores the unprimed responsiveness of the pituitary gland to the next pulse of GnRH. At the time of the LH surge, the action of GnRH (increased frequency and/or amplitude) is no longer sufficiently antagonized by GnSIF/AF (de Koning, 1995; Tijssen et al., 1997; de Koning et al., 2001).

Thus, GnSIF/AF acts in a functionally antagonistic way with GnRH to regulate pituitary LH secretion. Little is known about the intracellular interaction between GnRH and GnSIF/AF in the pituitary gland. Since GnRH increases MAPK activity, a step upstream from the synthesis

of SPAP, we investigated the possible involvement of this MAPK enzyme in the functional antagonistic control of LH release. In support of this model, the purified GnSIF/AF fraction and the SP-GnSIF/AF antagonised the GnRH activated MAPK activity in primary pituitary cells. All tests showed the strongest inhibition when the GnSIF/AF preparations were added during the last 5 min of a 15 min exposure to GnRH. These results are in line with those of Mitchell et al. (1994), who showed that GnRH-induced phosphorylation of MAPK started to increase only after a lag-phase of about 8 min. When GnRH and GnSIF/AF were added at the same moment, the inhibitory effect of GnSIF/AF was absent. GnRH must have terminated the action of GnSIF/AF via a separate transduction pathway during the lag-phase. No effect of GnSIF/AF was seen in the gonadotrophic αT3-1 cell line, suggesting that these cells do not express a functional GnSIF/AF receptor.

These are the first data obtained with purified GnSIF/AF preparations expressing inhibition of the intracellular signal transduction cascade initiated by GnRH. These data give further evidence to the posed functional antagonistic interaction between these (putative) hormones and their intracellular target in the normal pituitary cells. Thus, these results strongly indicate that GnSIF/AF exerts its inhibitory effect on the GnRH self-priming process at the level of MAPK or at processes upstream from MAPK.

Homology With Known Proteins

The amino acid sequences of the found GnSIF/AF proteins were coupled to an online Genbank to match with known proteins. High homology was found between the N-terminal sequence of the reduced HMW-GnSIF/AF band and human b-chain compliment C3. Thus, human b-chain compliment C3 or a homologous structure may be part of the HMW form of GnSIF/AF. During non-reduced PAGE, no bands with the same molecular weight of the whole complement C3 molecule could be detected (a-chain: 110 kDa, b-chain: 75 kDa and unprocessed precursor 187 kDa (Seya and Nagasawa, 1981)).

The N-terminal amino acid sequence of the LMW-GnSIF/AF protein showed high homology with other proteins, which seems to be ubiquitous in eukaryotes and prokaryotes. Since, all these approximately 40 kDa proteins (some with properties of receptor or signaling proteins) have the four conserved N-terminal Asp–Ile–Asn–Gly sequence, they are defined as members of the DING-family (Berna et al., 2002). One of these members, hSSP (205 kDa; three 70 kDa subunits), a T-cell attractant from rheumatoid arthritic synovial fluid or from the culture fluid of synovial fibroblasts, showed high homology with the GnSIF/AF sequence. This hSSP protein is an important auto-antigen sustaining the autoimmune inflammatory reaction in rheumatoid arthritis (Hain et al., 1996) and the 40 kDa DING-protein might be a proteolytic splice product

of hSSP (Adams et al., 2002). A similar DING-protein is produced in culture by normal and rheumatoid arthritis fibroblasts and by cervical carcinoma cells. Based on the molecular weight of hSSP and GnSIF/AF, physicochemical properties and composition, the proteins are most likely not identical.

Other human DING-family members include a 40 kDa p38 protein, isolated from human breast cancer MCF-7 cells (Belenky et al., 2003), and a 38 kDa human phosphate-binding protein (Diemer et al., 2008). Non-human examples of DING proteins are the bovine DING-protein presented here and a cotonine, a derivative of nicotine-binding protein isolated from rat brain (Belenky et al., 2003). Also, members from prokaryotes (proteins related to phosphate transport or metabolism) and plants are described (Berna et al., 2002; Pantazaki et al., 2008).

Surprisingly, none of the DING-protein members have corresponding genes annotated in genomic databases. Various techniques to find these sequences failed. Hence, the approach of designing relatively small, degenerate oligonucleotide primers to identify positive clones based on intermediate sequences proved to be unsuccessful by us (M.N. Helder and J. de Koning, results not shown). Also using the human articular cartilage cDNA of arthritic patients as a starting material (in the literature described to express hSSP) did not result in positive clones (M.N. Helder and J. de Koning, results not shown). So far, only one complete amino acid sequence of a DING-protein is known (hPBP; 376 amino acids) (Diemer et al., 2008). The meaning of the found homology in the conservative sequence amongst these DING proteins is not clear, and it is unknown to what extent the proteins are similar outside the N-terminal region. The peculiarity of DING genes also hampers further research.

The co-elution of GnSIF/AF bioactivity with inhibin bioactivity in bFF raises the question if the found proteins could be part of inhibin. Inhibin is a 32 kDa protein consisting of two subunits of 14 and 18 kDa. Both the molecular weight and the discovered amino acid sequence of GnSIF/AF are not comparable with inhibin, and therefore it is very unlikely that the found protein is related. Nevertheless, FSH suppression is seen in some assays and might be caused by other non-sequenced molecules from close fractions that also affect FSH. The produced SP-GnSIF/AF did not show FSH suppressive effects, which is an extra argument that the SP version shows true GnSIF/AF activity.

In conclusion, two new putative GnSIF/AF proteins (one might be a biologically active subunit of the other) have been purified from bFF, and a link was shown between purified protein bands after native PAGE and bioactivity. Moreover, these are the first data showing the influence of GnSIF/AF on an intracellular process involved in GnRH self-priming, and that the biological

action of GnSIF/AF was retained in the 26 amino acids encoded by a synthetic peptide. The two GnSIF/AF candidates show some homology with amino acid sequences from human proteins and bacteria. The results provide evidence that the discovered candidate protein is a true GnSIF/AF molecule.

MATERIALS AND METHODS

Source of Material

The ovaries of adult cows were removed immediately after death at the slaughterhouse (Schoonhoven, The Netherlands) and kept on ice until collection of the follicular fluid. bFF was aspirated from follicles 0.2–2.0cm in diameter. Charcoal (50 g/L; Sigma) was used to remove steroids from all preparations, including control sera (Koppenaal et al., 1992). The charcoal was subsequently removed by centrifugation at 10,000g. Estradiol, testosterone, and androstenedione concentrations in the supernatant were <200 pmol/L (assay detection limit) and the progesterone content was 3.3 nmol/L after charcoal treatment. The supernatant (600 ml) was used for further purification.

Bioassay of GnSIF/AF and MAPK-Assay

Animals

For bioassay purposes, female Wistar rats (24 days old) were purchased from Broekhuizen (Someren, The Netherlands) and were allowed free access to food and water in an animal room illuminated from 08.00 to 20.00 hr (summer time) and kept at a constant temperature of 22ºC. At the age of 28 days, the rats were sacrificed by decapitation. Animal procedures were in compliance with the Guidelines on the Handling and Training of Laboratory Animals (Universities Federation for Animal Welfare) and the local Institutional Animal Care Committee.

Bioassay

Rat pituitary monolayer cell culture was used for bioassay of GnSIF/AF and inhibin. Anterior pituitaries were dispersed by trypsin and EDTA treatment into single cells (Denef et al., 1978; Koppenaal et al., 1992). The cells were suspended in a tissue culture medium, transferred to 24-multiwell dishes (NUNC, Copenhagen, Denmark) (100,000–200,000 cells/well) and incubated in a humidified CO2-air incubator (1.2% CO2) at 37ºC. The culture medium, without phenol red, was essentially the same as previously described (Baes and Denef, 1987). Dexamethasone (4 nmol/L; Organon, Oss, The Netherlands) and 3,3”,5-tri-iodo-L-thyronine (50 pmol/L; Sigma Chemie, Axel, The Netherlands) was added to improve the attachment of the cells. Steroid-free newborn calf-serum (Flow laboratories Ltd., Rickmansworth, Herts, UK) was added in a final concentration of 10%, as it increases the reproducibility of the LH response to GnRH (Koppenaal et al., 1992).

Cells were incubated as monolayer in 0.5 ml culture medium and exposed to the samples. Inhibition of basal FSH release after adding the various samples was used as a measure for inhibin bioactivity (Danforth et al., 1987). After 20 hr of incubation, the culture medium was replaced with 1ml culture medium TC 199 (Boehringer, Mannheim, Germany) containing 1 mmol/L GnRH (Boehringer), and the cells were incubated for 45 (LH lag-phase response) or 90 min (protein synthesis-dependent increased LH response) at 37ºC with continuous shaking (30 rpm) in an atmosphere of 95% O2 and 5% CO2. This concentration of GnRH is equally effective as the just maximally effective concentration of GnRH (1 nmol/L). The high GnRH concentration was used to prevent GnRH from becoming a rate limiting factor in the LH secretion process. We have shown that this protocol can be used to demonstrate that inhibition of GnRH-stimulated LH secretion during this incubation period might be an index for GnSIF/AF bioactivity (Tio et al., 1998). After pre-incubation in medium only (collected for estimation of FSH release) and the subsequent incubation with GnRH (collected for estimation of LH release), samples of medium were taken, centrifuged to remove possible attached cells (733g for 10 min) and the respective FSH or LH concentrations were measured by radioimmunoassay (Tio et al., 1998).

Purification Design

Preparation of bovine follicular fluid for chromatographic techniques

Stepwise ammonium sulfate precipitation (saturation increased by steps of 20%) of steroid-free bFF was performed according to the protocol of Green and Hughes (1955). The only adaptation was that the procedure was carried out on ice, for which the amount of salt was reduced to one tenth (Dr. R. Amons, Protein Sequencer Facility, Sylvius Laboratory, Leiden; personal communication). After incubation for 30 min, a saturated solution was centrifuged for 30 min at 4ºC in a Sorvall GSA rotor at 10,000g. The pellets were dissolved in 20 mmol/L Tris–HCl (pH 7.5). The total protein content of the pellet fractions and their relative bioactivities were measured. Small aliquots of the purified fractions were dialysed against the Tris–HCl buffer prior to testing in the bioassays.

Chromatographic techniques

The eluted fractions were screened for their bioactivity. In each case biologically active pooled fractions of the previous purification step were loaded on the columns. The chromatographic techniques were applied in the order described below.

Hydrophobic interaction chromatography (HIC)

After dialysis against 20 mmol/L Tris–HCl (pH 7.5), the 20–40% ammonium sulfate precipitated fraction was applied twice on a 200 ml Phenyl Sepharose® HIC column (Pharmacia). The second time, the collected fraction was diluted by half of its volume with the Tris–HCl buffer and the column was washed with 400 ml of this buffer. This procedure resulted in flow through of a large

percentage of protein, thereby increasing the capacity and specificity of the column. Elution was performed at gravity speed using a linear gradient: 20 mmol Tris–HCl/20 mmol/L Tris–HCl + 80% ethylene glycol (pH 7.5; 75/75 ml). Finally, the column was eluted with the same buffer, but now with 90% ethylene glycol. Ten milliliter fractions were collected.

Fast protein liquid chromatography (FPLC) gel filtration

The bioactive fractions were dialysed against several changes of 1 mmol/L Tris–HCl (pH 7.4), concentrated and loaded in 3ml portions onto a Hiload Superdex 200® FPLC column (Pharmacia). Elution took place with 20 mmol/L Tris–HClþ60 mmol/L NaCl, pH 7.4, being the starting buffer of the next chromatographic step. The elution speed was 0.07 ml/min and 1ml fractions were collected.

Anion exchange chromatography (AEC)

The bioactive fractions were loaded onto a FPLC Mono-Q® column (Pharmacia). The column was washed with 20 mmol/L Tris–HCl + 60 mmol/L NaCl (pH 7.4). Bound proteins were eluted with a linear gradient from 60 to 600 mmol/L NaCl in the same buffer. The elution speed was 0.5 ml/min and 0.5 ml fractions were collected.

High performance liquid chromatography (HPLC) gel filtration

The bioactive fractions were further separated by HPLC gelfiltration using a Shodex 800® column (Pharmacia). The fractions were eluted with 20 mmol/L Tris–HCl + 60 mmol/L NaCl (pH 7.4). The elution speed was 0.4 ml/min and 0.2 ml fractions were collected.

Analysis of purified fractions

Standard or reducing SDS–PAGE was performed to determine the molecular weight of proteins in the various purified fractions. Native PAGE was performed prior to determining the bioactivity of the protein bands. The samples were loaded in duplicate on adjacent lanes. After running the gel, one of the lanes was stained and used to localize the corresponding bands in the unstained lane. The unstained bands were collected separately and were used in the bioassay after overnight diffusion of the protein material in assay medium. The stained bands, after being soaked in reducing SDS-buffer, were loaded on SDS–PAGE for estimation of the molecular weight of the corresponding protein band. In this way, biological activity and molecular weight of the proteins could be matched.

The protein bands after reduced SDS–PAGE were electroblotted onto a polyvinylidene difluoride membrane (PVDF; Immobilon-P) for amino acid sequence analysis. The protein sequence analysis was performed by Dr. R. Amons in the Protein Sequencer Facility, Leiden, using a Hewlett–Packard G1004A protein sequencer system. A 26 residue peptide was synthesized

from the determined N-terminal sequence of the GnSIF/AF protein. The effect of this SP-GnSIF/AF was tested in a GnSIF/AF bioassay and used in the MAPK experiments (see below).

MAPK-assay

Homologous mouse gonadotrophic αT3-1 cell cultures, an immortalized gonadotrophic cell line (a generous gift from Dr. P.L. Mellon) or primary pituitary cells derived from female rats were seeded in six-well plates (400,000 cells/well) and incubated for 24 hr in serum-free medium to reset MAPK levels to baseline values. The gonadotrophic αT3-1 and primary pituitary cells were incubated for 15 min with 1 mmol/L GnRH. GnSIF/AF preparations were added at 0, 5, and 10 min after the start of GnRH stimulation (0.3 mmol/L of SP-GnSIF/AF or various amounts of highly purified bovine GnSIF/AF) (Mitchell et al., 1994). Thus, GnRH was present throughout the 15 min incubation period. After incubation, the cells were lysed in an SDS–PAGE buffer. The lysates were fractionated on 8.5% SDS–PAGE and MAPK activation was quantitated on Western blots by determining the optical densities of the protein bands MAPK (Erk1) and MAPK-P (migrating slightly

higher than MAPK). MAPK activity is calculated as the percentage of MAPK-P of total MAPK (Erk1 plus MAPK-P). Every MAPK assay included control samples consisting of vehicle only or contained phorbol 12-myristate 13-acetate (PMA, 10 mmol/L) to show maximal stimulation. This procedure has been described before (van der Zon et al., 1996; Telting et al., 1999). The final results are expressed as a percentage increase above vehicle values. The experiments were carried out at least on three separate occasions.

Matching GnSIF/AF

The amino acid sequence of the putative GnSIF/AF proteins were coupled to the online Genbank (www.ncbi.nlm.nih.gov/Genbank/index.html) to match with known protein sequences.

Statistical Analysis

Statistical comparisons were made by analysis of variance and by Duncan's multiple comparison test. If the data showed heterogeneity of variance, logarithmic transformation was carried out before the analysis. A difference was considered significant when analysis of variance showed significant heterogeneity among all groups and the multiple comparison test gave a value of $P<0.05$ for the groups concerned.

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