Evidence that progesterone binding uteroglobin is similar to myosin alkali light chain

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Using a computer program designed to detect evolutionary relationships between proteins, I find that exon 2 of rabbit uteroglobin, a progesterone binder, and part of myosin alkali light chain have a comparison score that is 7.2 standard deviations higher than that obtained with a comparison of randomized sequences of these proteins. The probability (p) of getting this score by chance is less than 10^{-12}. This theoretical finding that these sequences are similar has led to the experimental finding that copper, calcium and the tranquilizer trifluoperazine, a calmodulin binding ligand, affect progesterone binding to uteroglobin.

Calcium-modulated protein *Myosin* *Uteroglobin* *Progesterone* *Copper* *Prostatic steroid binding protein*

1. INTRODUCTION

Myosin alkali light chain, troponin C, parvalbumin and calmodulin belong to a family of calcium-modulated proteins [1–5]. These proteins have no apparent functional relationship to steroid hormone binding proteins such as rabbit uteroglobin [6–10] and rat prostatic steroid binding protein [11–14]. Serendipitously, I discovered that the primary amino acid sequences of uteroglobin and prostatic steroid binding protein were similar to those of calcium-modulated proteins while exploring the hypothesis that steroid hormone binding proteins, including uteroglobin and prostatic steroid binding protein, contain some chemical structures that resemble those found in serine proteases [15–17]. That hypothesis led me to compare the amino acid sequences of several steroid hormone binding proteins with those of serine proteases, using computer programs developed for that purpose at the National Biomedical Research Foundation [18–22]. As a control for those comparisons, I compared rabbit uteroglobin with calmodulin and unexpectedly found that the primary amino acid sequences of these 2 proteins were similar. More comprehensive computer-based analyses of uteroglobin and prostatic steroid binding protein with other calcium-modulated proteins showed that among these latter proteins, myosin alkali light chain 1 of rabbit fast skeletal muscle is most similar to rabbit uteroglobin and troponin C is most similar to rat prostatic steroid binding protein. These theoretical results stimulated me to search for other similarities between uteroglobin and calcium-modulated proteins, which led to the novel experimental finding that copper, calcium, and the tranquilizer trifluoperazine, a calmodulin binding ligand, affect progesterone binding to uteroglobin. Here I report the details of these theoretical and experimental studies.

2. MATERIALS AND METHODS

2.1. *Computer analyses*

The Relate program compares all possible segments of a given length from one sequence with all segments of the same length from a second sequence [18–22]. A segment score is accumulated from the pair scores of the amino acids occupying corresponding positions with the 2 segments. The pair scores are specified using an empirically de-

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rived mutation data matrix [18–22]. The mean of a number of highest scores is determined for the given sequences and for 250 comparisons of random permutations of the sequences. The segment comparison score is calculated as the difference between the mean of the real sequences and the averaged value determined from the randomized sequences divided by the standard deviation (SD) of the values of the randomized sequences. The segment comparison score is thus expressed in SD units. A score greater than 5 SD units (p less than 2.8×10^{-7}) indicates that the sequences of the 2 proteins are similar and this suggests that the proteins may also have similarities in their biological activities. Scores between 3 SD ($p < 10^{-3}$) and 5 SD ($p < 2.8 \times 10^{-7}$) support a relationship between the sequences of the 2 proteins if there are other indications such as similarity of function.

2.2. *Progesterone binding to rabbit uteroglobin*

Uteroglobin was extracted from rabbit uteri by homogenization with 1 g tissue/4 ml buffer with a polytron homogenizer. The homogenate was centrifuged first for 15 min at 10000 × g and then the resulting supernatant was centrifuged for 1 h at 190000 × g. This cytosol was partially purified by filtration on Sephadex G-75, which separated uteroglobin from various proteins including corticosterone binding globulin and progesterone receptor [7,8]. The binding of progesterone to uteroglobin was determined by incubation of uteroglobin at pH 7.8 in buffer consisting of 50 mM Hepes, 50 mM NaCl, 10 mM dithiothreitol, and 6.3×10^{-8} M H-progesterone for 15 min at 37°C. Then aliquots of the sample were added to tubes containing various compounds or 3×10^{-5} M unlabeled progesterone (to determine nonspecific binding). These samples were incubated for 1 h at 22°C and then for 1 h at 0°C. Bound steroid was determined using a dextran-coated charçoal technique similar to that described by Beato [23]. Each sample was done in duplicate. Variation was less than 10%. The stock copper chloride solution contained nitrilotriacetic acid at 10% molar excess. Nitrilotriacetic acid is a 'soft' chelator that prevents polymerization of the copper ion [24].

Table 1

Segment comparison scores of exon 2 of rabbit uteroglobin and calcium-modulated proteins

Protein	Score (SD)	p
Rabbit myosin alkali light chain (residues 109–168)	7.2	$<10^{-12}$
Chicken myosin alkali light chain (residues 108–167)	6.6	4×10^{-11}
Calmodulin (residues 1–51)	4.5	3.4×10^{-6}
Rabbit troponin C, skeletal muscle (residues 1–58)	4.2	10^{-5}

These segment comparison scores were obtained using the Relate program (segment length 20 amino acids) and 250 random permutations of the sequences for statistical analysis

3. RESULTS AND DISCUSSION

3.1. *Some properties of uteroglobin and prostatic steroid binding protein*

Rabbit uteroglobin consists of 2 identical polypeptides (M_r ~8000) joined by 2 disulfide bridges [25–27]. Progesterone both induces uteroglobin synthesis in rabbit uterus and binds to uteroglobin (K_d ~1 μM). Rat prostatic steroid binding protein (PSBP) is a tetramer consisting of 2 subunits: an A subunit containing polypeptides C_1 (M_r ~10200) and C_3 (M_r ~8600) and a B subunit containing C_2 (M_r ~10500) and C_3 [28–31]. Androgens both induce prostatic steroid binding protein synthesis and bind to prostatic steroid binding protein (K_d ~1 μM). Uteroglobin and PSBP's C_1 and C_2 polypeptides exhibit amino acid sequence homology [32] and similar gene structures (M. Beato and M. Parker, personal communication). Because synthesis of uteroglobin and PSBP is induced by steroids, these proteins are useful model systems for studying gene regulation, in addition to their importance in reproductive biology.

Myosin alkali light chain, troponin C and parvalbumin were originally found in muscle cells. In the last few years these proteins have been found in many other types of cells. Most of these proteins bind calcium and/or participate in processes that are calcium regulated. Calcium-modulated proteins appear to have evolved from an ancestor of about 40 residues by gene duplication [1–4,33].

3.2. *Computer-based similarity between uteroglobin and myosin alkali light chain*

A preliminary computer analysis using the Relate program [18–22] indicated that residues 1–60 (exon 2) of uteroglobin [27,34] were similar to myosin alkali light chain. Table 1 summarizes the results of a Relate analysis of exon 2 of uteroglobin with myosin alkali light chain, calmodulin and troponin C, and in parentheses the regions in these proteins that have the closest relationship to exon 2 of uteroglobin are described. The Relate analysis comparison of exon 2 of uteroglobin and rabbit myosin alkali light chain gives a score that is 7.2 SD higher than the score found with randomized sequences of these proteins. The probability (p) of getting a score of 7.2 SD by chance is $< 10^{-12}$. The alignment of residues 1–60 (exon 2) of uteroglobin and residues 109–168 of the myosin alkali light chain is shown in fig.1. Based on Nabeshima et al. [36] report of the gene structure of myosin alkali light chain in chicken, fig.1 shows an alignment of part of exons 6 and 7 of myosin alkali light chain with exon 2 of uteroglobin.

3.3. *EF-hand structure analysis of uteroglobin*

To further explore the relationship between uteroglobin and calcium-modulated proteins, exon 2 of uteroglobin was analyzed in terms of the EF-hand structure. This structure has been useful in elucidating how calcium-modulated proteins bind calcium [3,35]. Fig.1 shows the nomenclature of the EF-hand superimposed upon the alignment of uteroglobin and myosin alkali light chain. As

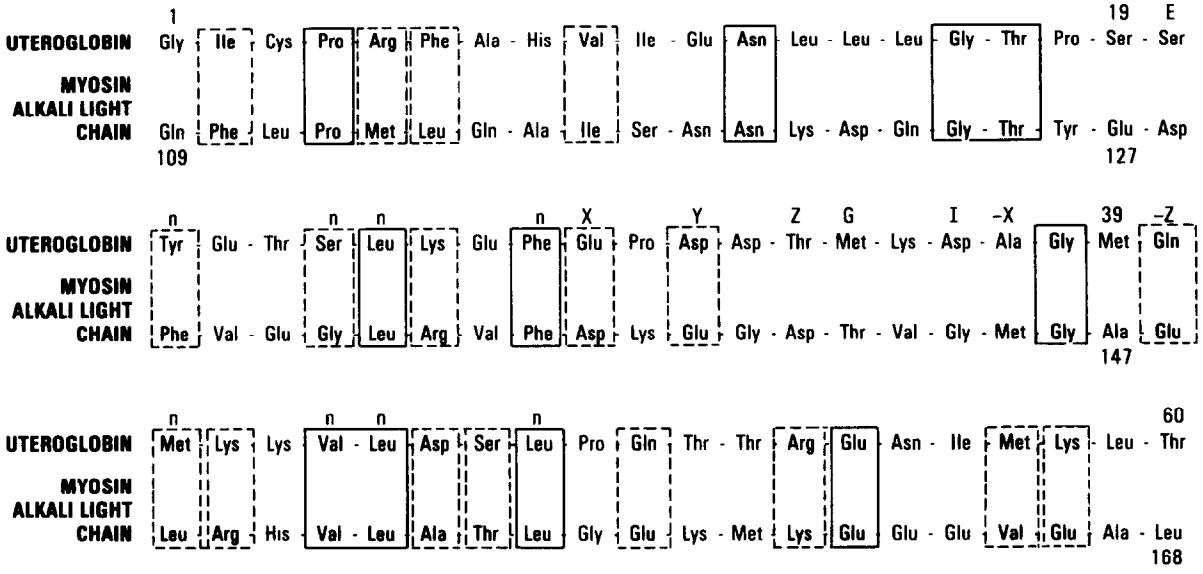

Fig.1. Alignment of residues 1–60 of rabbit uteroglobin and residues 109–168 of rabbit myosin alkali light chain. Residue numbering refers to the mature sequence of these proteins. Solid boxes show identities and the dotted boxes show replacements that both have a single nucleotide difference and are frequently observed in families of proteins. The EF-hand model developed by Kretsinger [3,35] for analyzing the calcium binding domain of calcium-modulated proteins is notated above the uteroglobin sequence. α-Helix E starts at residue 20. The position of hydrophobic residues that are conserved at the interior of the α helix are notated with n. The positions where amino acid side chains provide oxygen to coordinate with calcium are notated X, Y, Z, $-$X and $-$Z. Amino acids that can do this are Asp, Asn, Glu, Gln, Ser or Thr. An invariant glycine following Z and isoleucine preceding $-$X are also shown. In this EF-hand domain, the uteroglobin sequence at X, Y, Z and $-$Z has oxygen ligands that could coordinate with calcium. At the hydrophobic positions of the α helix, uteroglobin has amino acids that are either identical to or conservative replacements of corresponding residues on myosin alkali light chain. The alanine residue at $-$X would not provide an oxygen to coordinate with Ca^{2+}. This and the absence of the glycine and isoleucine residues, which are conserved in calcium binding EF-hand domains, in uteroglobin make it unlikely that this protein has a high affinity for calcium.

shown in fig.1 the amino acid residues at X, Y, Z, −Z of uteroglobin have oxygen containing side chains that can coordinate with calcium. Also, fig.1 shows that both proteins lack an oxygen-coordinating residue at the −X coordinate and the glycine and isoleucine residues that are conserved in EF-hand domains that bind calcium with high affinity [3,35]. The positions in the interior of the 2 α helices on uteroglobin that have hydrophobic residues are either identities or conservative replacements of corresponding residues in myosin alkali light chain. These correspondences in the EF-hand structures of uteroglobin and myosin alkali light chain provide additional evidence that these proteins are similar. Myosin alkali light chain does not bind calcium with high affinity even though it is homologous to proteins that do. The EF-hand analysis predicts that uteroglobin would not have a high affinity for calcium. Since the gene for uteroglobin has been cloned [27,34], it is possible to use recombinant DNA techniques to construct a uteroglobin molecule with residues that fulfill the EF-hand requirements for binding calcium with high affinity. It would be interesting to know if this uteroglobin variant bound calcium and progesterone.

The comparison score of 7.2 SD ($p < 10^{-12}$), which was obtained from the computer analysis of the primary amino acid sequences of uteroglobin and myosin alkali light chain, suggests that these 2 proteins are similar. The EF-hand structure in these proteins is also similar. These analyses do not determine whether these proteins are derived from a common ancestor or whether the similarities between uteroglobin and myosin alkali light chain are the result of convergent evolution. However, in either case, the similarities between these proteins have sufficient statistical substance to suggest additional computer analyses and experimental studies, which have interesting implications for the evolution and function of uteroglobin and rat prostatic steroid binding protein.

3.4. *Computer-based similarity between the C3 chain of rat prostatic steroid binding protein and troponin C*

Because rabbit uteroglobin is related to the C_1 and C_2 chains of rat prostatic steroid binding protein [32], it seemed useful to compare the amino acid sequences of C_1 and C_2, with those of calcium-modulated proteins. C_3 was used as a control because its amino acid sequence does not show similarity with C_1 and C_2 [32]. The Relate analysis comparison scores for C_1 and C_2 with parvalbumin were at the borderline for significance. For example, the Relate comparison of C_1 and rabbit parvalbumin gave a score of 3 SD. The other calcium-modulated proteins had lower scores. Unexpectedly, the amino acid sequence of 'the control protein' C_3 [29,37,38] appeared to be similar to that of frog troponin C (6.5 SD, $p = 4 \times 10^{-11}$). As would be expected, C_3 shows some similarity to other calcium-modulated proteins. Table 2 summarizes the results of the comparisons of C_3 with different calcium-modulated proteins. The finding that both uteroglobin and C_3 have amino acid sequences that are similar to calcium-modulated proteins and the similarity among uteroglobin, C_1 and C_2 suggests that uteroglobin, C_1, C_2 and C_3 have evolved from a common ancestor. Interestingly, Parker et al. [37,38] proposed that C_3 was related to C_1 and C_2 on the basis of similarities of their gene structure. Further, W. Barker (personal communication) has found that a computer analysis with the Align program of the entire uteroglobin and C_3 amino acid sequences (including the signal peptide) indicate that these proteins are similar. W. Heyns (personal communication) reached a similar conclusion. Their findings and the results reported here support the proposal of Parker et al. that C_1, C_2 and C_3 evolved from a common ancestral gene.

Table 2

Segment comparison scores of exon 2 of C_3 chain of rat prostatic steroid binding protein and calcium-modulated proteins

Protein	Score (SD)	p
Frog troponin C (residues 55–117)	6.5	4×10^{-11}
Calmodulin (residues 45–107)	4.8	10^{-6}
Rabbit myosin alkali light chain (residues 9–71)	4.0	3×10^{-5}
Thornback ray parvalbumin (residues 23–85)	4.2	10^{-5}

These segment comparison scores were obtained using the Relate program (segment length 30 amino acids) and 250 random permutations of the sequences for statistical analysis

Uteroglobin and prostatic steroid binding protein belong to a family of proteins whose functions are still being elucidated [32,39]. Uteroglobin is important in reproductive processes [9,10,40]. Estrogen mustards, which bind to prostatic steroid binding protein, are used in treating cancer of the prostate [41,42]. Like other steroid hormone binding proteins, uteroglobin and prostatic steroid binding protein contain a nucleophilic site that both recognizes inhibitors and substrates of serine proteases and influences steroid hormone binding (M. Baker, unpublished). The function of this site is not established. The similarity between the amino acid sequences of uteroglobin, prostatic steroid binding protein and calcium-modulated proteins presented in tables 1 and 2 suggests some interesting experiments that could provide clues for elucidating the functions of these proteins. These experiments include determining if uteroglobin and prostatic steroid binding protein bind calcium or other metal ions or if phosphorylation of uteroglobin and prostatic steroid binding protein is important in their functioning. On the other side of the coin, it would be interesting to determine if steroid hormones and/or electrophilic compounds that are inhibitors and substrates of serine proteases interact with calcium-modulated proteins, even though these proteins are unlikely to have the same functions as uteroglobin and prostatic steroid binding protein.

3.5. *Effect of some divalent metal ions on progesterone binding to uteroglobin*

To begin to explore these possibilities, the effect of divalent metal ions such as calcium, magnesium and copper on progesterone binding to rabbit uteroglobin was studied. Studies from Beato's laboratory [23,43,44] show that treatment of uteroglobin with agents such as dithiothreitol, to reduce the 2 disulfide bridges in uteroglobin, increase its affinity for progesterone by over 10-fold compared to the oxidized dimer. Also, it appears that the steroid binding species of uteroglobin is a tetramer [44]. As shown in table 3a, neither 5 mM calcium nor 5 mM magnesium had an effect on progesterone binding to uteroglobin. Unexpectedly, 0.5 mM $CuCl_2$ complexed with 0.55 mM nitrilotriacetic acid, a soft chelator [24], increased specific [^{3}H]progesterone binding to uteroglobin from 10000 to 40000 cpm/ml (table 3a). Copper interacts with a histidine residue on albumin to promote its polymerization [45]. Analogously, copper could interact with histidine and/or cysteine on uteroglobin to stabilize the progesterone binding conformation. Uteroglobin in the presence of copper appears to bind calcium and magnesium with low affinity. Adding 5.0 mM $CaCl_2$ to the sample with 0.5 mM $CuCl_2$ reduces specific [^{3}H]progesterone binding to uteroglobin to 18000 cpm/ml (table 3b). And when $MgCl_2$ is substituted for $CaCl_2$ in this experiment, specific [^{3}H]progesterone binding is decreased to 33000 cpm/ml. Thus calcium is more effective than magnesium in antagonizing the effect of $CuCl_2$ on progesterone binding to uteroglobin. This suggests that there is a specificity for calcium in its interaction with uteroglobin. This relatively low affinity of calcium for uteroglobin, at least compared to troponin C, calmodulin and parvalbumin, is what would be expected from the EF-hand analysis of uteroglobin (fig.1).

The tranquilizer trifluoperazine binds to calmodulin with μM affinity [45]. Resins with covalently attached trifluoperazine are used to purify calmodulin by affinity chromatography. Trifluoperazine inhibits progesterone binding to uteroglobin (table 3b). The binding of trifluoperazine by uteroglobin provides another similarity between this protein and calmodulin.

The findings presented in table 3 showing that copper, calcium and trifluoperazine influence progesterone binding to uteroglobin may have biological implications. In particular the possibility that copper intrauterine devices could supply copper ions that could interact with proteins from the uterus and semen that are related to uteroglobin should be considered. In this regard, it is interesting that in 1976 Daniel reported that neutron-activation analysis of electrophoretically purified uterine washings showed that copper was preferentially associated with a uteroglobin containing fraction [10].

Thus, the computer analysis, which shows that exon 2 of uteroglobin is similar to calcium-modulated proteins, has led to experiments showing that metal ions and trifluoperazine affect the progesterone binding to uteroglobin. It will be important to see whether the other questions raised from the computer analysis reported here also yield interesting experimental findings.

Table 3

Effect of metal ions and trifluoperazine on [^{3}H]progesterone binding to rabbit uteroglobin

Sample	Specifically bound [^{3}H]progesterone (cpm/ml)	% of control	% of 0.5 mM $CuCl_2$ sample
(a)			
Control	9730	100	
5.0 mM $CaCl_2$	9780	100	
5.0 mM $MgCl_2$	9070	93	
12.5 μM trifluoperazine	7685	79	
1.0 mM $CuCl_2$	36835	375	92
0.5 mM $CuCl_2$	40225	410	100
0.25 mM $CuCl_2$	33315	340	83
(b) Incubated with:			
0.5 mM $CuCl_2$ and 5.0 mM $CaCl_2$	17645	180	44
5.0 mM $MgCl_2$	33070	340	82
12.5 μM trifluoperazine	28360	290	70
12.5 μM trifluoperazine + 5 mM $CaCl_2$	11600	120	29

Uteroglobin was incubated at pH 7.8 in buffer consisting of 50 mM Hepes, 50 mM NaCl, 10 mM dithiothreitol and 6.3×10^{-8} [^{3}H]progesterone for 15 min at 37°C. Then aliquots of the sample were added to tubes containing various compounds, or 3×10^{-5} M unlabeled progesterone (to determine non-specific binding). These samples were incubated for 1 h at 22°C and then for 1 h at 0°C. Bound steroid was determined using a dextran-coated charcoal technique similar to that described by Beato [23]. Each sample was done in duplicate. Variation was less than 10%. The stock copper chloride solution contained nitrilotriacetic acid at 10% molar excess. Nitrilotriacetic acid is a 'soft' chelator that prevents polymerization of the copper ion [24]

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