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Crosslinking of Uteroglobin by Transglutaminase

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SUMMARY: Uteroglobin, a progesterone induced, pregnancy related protein, can be incorporated into higher molecular weight proteins by human placental Factor XIIIa. This process is time dependent, requires $CaCl_2$ and can be inhibited by the addition of polylysine, dansylcadavarine or histamine. Crosslinking of uteroglobin into higher molecular weight proteins can also be brought about by guinea pig liver transglutaminase. Such a process may be involved in the modification of epididymal spermatozoa to suppress their antigenicity.

Uteroglobin (1) or blastokinin (2) is a progesterone induced, pregnancy related protein synthesized by the rabbit endometrial cells. It is a small molecular weight protein (M.W. 15,000 daltons) with two identical subunits joined together by two interchain disulfide bonds. In addition to the pregnant rabbit uterus, uteroglobin has also been detected in the seminal vesicle (3) and the lung (4). Evidence for a specific function for this protein is fragmentary. It has the ability to bind steroids (5). Although this protein was proposed to be necessary for the growth of the rabbit embryo (6) a contradictory report has been published (7).

Transglutaminase is a Ca^{++} dependent enzyme (8) that can mediate the crosslinking of proteins like HLA-A and -B (9), β_2-microglobulin (10), fibrin (11) and collagen (12). Crosslinking of cell surface sites may be involved in Ca^{++} mediated crosslinking of erythrocyte proteins (13)

Abbreviations: Factor XIIIa, Factor XIII activated by thrombin; BSA, Bovine Serum Albumin; MDC, Mono Dansylcadavarine; PBS, Phosphate Buffered Saline; SDS, Sodium Dodecyl Sulfate; PMSF, Phenyl Methyl Sulfonyl Flouride; EDTA, Ethylene Diamine Tetra Acetate; DTT, Dithiothreitol; UTG, Uteroglobin; TCA, Trichloroacetic acid.

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lymphocyte activation (14), and receptor mediated endocytosis (15). Evidence has been presented which suggests the involvement of at least two factors, uteroglobin and transglutaminase (coagulation factor XIIIa; R-glutaminyl-peptide: amine γ-glutamyltransferase, EC 2.3.2.13) in masking *in vitro* the antigenicity of embryonic cells (16) as well as epididymal spermatozoa (17). The uterus contains an enzyme which is apparently identical to the placental enzyme (18). Hence the present work was undertaken to delineate whether uteroglobin could participate in a placental Factor XIIIa catalysed covalent attachment to other proteins or cellular surface sites.

Materials and Methods:

(a) Proteins and inhibitors: Enriched human placental, Factor XIII was a gift from Dr. I. V. Brock of Berhrinwerke, Marburg, Germany. The lyophilised Factor XIII was dissolved in PBS and the activity as measured by ^{14}C-amine incorporation into casein (19) was adjusted to 5 units/ml. The enzyme was stabilised by the addition of BSA. Guinea pig liver transglutaminase was purified according to Connellan *et al.* (20). The purified protein had a specific activity of 23 units/mg protein as assayed by hydroxyamate formation with benzyloxy carbonyl (2) L-glutaminyl glycine. Uteroglobin was purified from pregnant rabbit uterine flushings according to Nieto *et al.* (1) with modifications (16). Antibody to uteroglobin used during purification of this protein is a generous gift from Dr. David Bullock of Baylor College of Medicine, Houston, Texas. The purified protein gave a single band when electrophoresed in 15% urea SDS gels according to Swank and Munkres (21). Purified samples (10 µg) of uteroglobin were iodinated by a modified chloramine-T method (22). MDC, polylysine (mol. wt. 90,000) and histamine were obtained from Sigma Chemical Co., St. Louis, Mo. Sperm were isolated from rabbit epididymis by squeezing them into PBS and spun down at 800 X g for 10 mins. They were washed twice with PBS containing PMSF (0.1mM) before being resuspended in the same solution.

(b) Crosslinking of uteroglobin by transglutaminase: The reaction mixture consisted of 30mM Tris-HCl buffer pH 7.2, 1mM EDTA, 10mM $CaCl_2$, 3.8mM DTT, 1 x 10^6 cpm of iodinated uteroglobin, various amounts of cold uteroglobin and transglutaminase in a total volume of 100 µl. When placental factor XIII was used, the reaction mixture contained, in addition to all the above mentioned components, 2.5 units of thrombin. Incubating at 37°C for 1 hr, 30 µl aliquots of the mixture were removed and mixed with SDS sample buffer. The samples were heated at 90°C for 2 minutes and electrophoresed on 10% SDS polyacrylamide slab gels according to Laemmli (23). Following electrophoresis the gels were fixed in 10% TCA, washed with water and autoradiographed after wrapping the wet gel in Saran wrap. Exposure was carried out for 12 to 18 hours in Kodak X-Omat cassettes using Kodak X-Omat [XAR-5] film. Gels were also stained with Coomassie Blue R250 and destained for direct visualization of the protein bands. Iodoacetamide inactivation was carried out in 0.1 M Tris HCl buffer pH 7.5 with 1.2 nmoles of guinea pig liver transglutaminase, 12 nmoles of iodoacetamide and 1µ mole $CaCl_2$. The mixture was incubated at 37°C for 1 hr and the inactivation stopped by adding an excess of DTT.

Results

Figure 1 demonstrates the action of human placental Factor XIII on [^{125}I] uteroglobin. Incubation of uteroglobin with 0.05 units placental Factor XIIIa results in incorporation of [^{125}I] uteroglobin into higher molecular weight proteins (Fig. 1B). These proteins appear to have molecular weights higher than 95,000 and do not enter the resolving gel (Fig. 1A). Omission of $CaCl_2$ from the reaction mixture abolished this

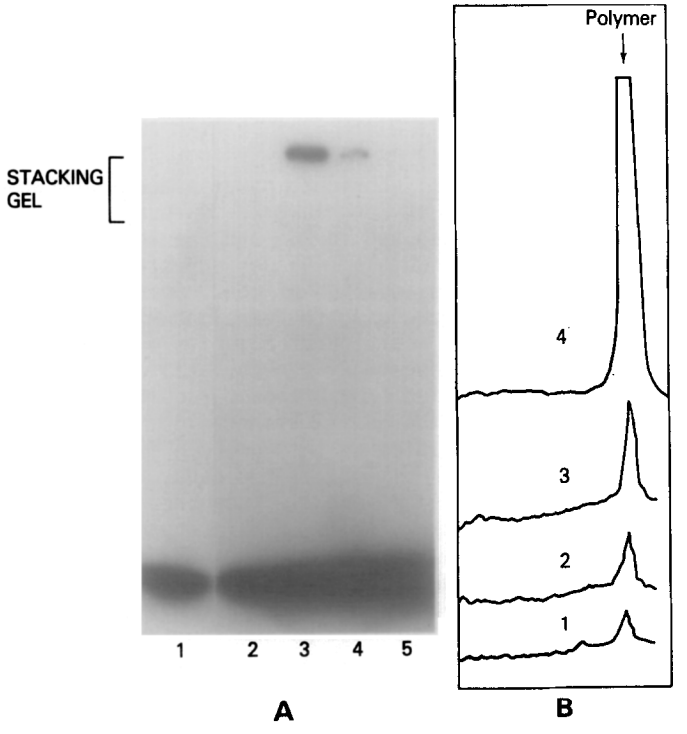

Fig. 1A. Crosslinking of uteroglobin by human placental Factor XIII. 15 µg of uteroglobin was incubated with 0.7 unit of Factor XIIIa at 37°C for 1 hr. The reaction was done as given in "Materials and Methods". Proteins were separated on 10% SDS polyacrylamide gels and autoradiographed. Lane 1: [^{125}I] uteroglobin alone; Lane 2: Uteroglobin incubated without enzyme. Lane 3: Uteroglobin and enzyme in complete reaction mixture. Lane 4: Uteroglobin incubated with enzyme in the absence of DTT. Lane 5: Uteroglobin incubated with enzyme in the absence of $CaCl_2$.

1B. Crosslinking was done as given in "Materials and Methods". 150 µg of uteroglobin was incubated for 1 hr at 37°C with 0.05 unit (tracing 1), 0.1 unit (tracing 2) and 0.2 units (tracing 3) human placental Factor XIIIa. Proteins electrophoresed in the absence of stacking gels were autoradiographed and the exposed film was scanned densitometrically. Tracing 4 shows polymer formation at 18 hrs of incubation with 0.1 units. The free uteroglobin peak has not been shown.

conversion. The process was time dependent and incubation with increasing concentrations of the placental enzyme leads to formation of increasing amounts of high molecular weight proteins (Fig. 1B). Uteroglobin when incubated alone did not result in any conversion. Incubation of [^{125}I] myoglobin with Factor XIIIa alone also did not result in any conversion (Fig. 4B).

Transglutaminase catalyses a Ca^{++} dependent acyl transfer reaction in which the γ-carboxamide groups of peptide bound glutamine residues are the acyl donors. Primary amino groups in a variety of compounds may act as the acyl acceptors. [^{3}H] putrescine and MDC were used to examine the substrate properties (acyl donor or acceptor) of uteroglobin. No incorporation of either radioactivity or fluorescent MDC into uteroglobin was observed even at excess concentration of amines. However, other primary amine containing compounds like polylysine (10 μM), histamine (0.6 mM) and MDC (0.5mM) caused inhibition of uteroglobin conversion into higher molecular weight proteins (Fig. 2) These results suggest that uteroglobin interacts with Factor XIIIa as an acyl acceptor substrate. It is likely that uteroglobin is incorporated preferentially into self polymerised Factor XIIIa, thus not entering the resolving gel.

Incubation of uteroglobin with another class of transglutaminase present in the epithelial cells of uterine endometrium (24) also results in incorporation of [^{125}I] uteroglobin into high molecular weight proteins (Fig. 3). Incubation of uteroglobin with iodoacetamide-inactivated transglutaminase abolished the reaction completely (data not shown).

In order to test the possible involvement of uteroglobin in tne covalent modification (attachment) of cellular protein, rabbit epididymal spermatozoa were employed as acyl donors. Using indirect immunofluorescent techniques it has been recently demonstrated that uteroglobin in the prostatic fluid and transglutaminase may interact on the sperm surface bringing about suppression of the antigenicity of spermatozoa (17). Incubation of rabbit epididymal sperm with Factor XIIIa in presence of [^{125}I] uteroglobin but

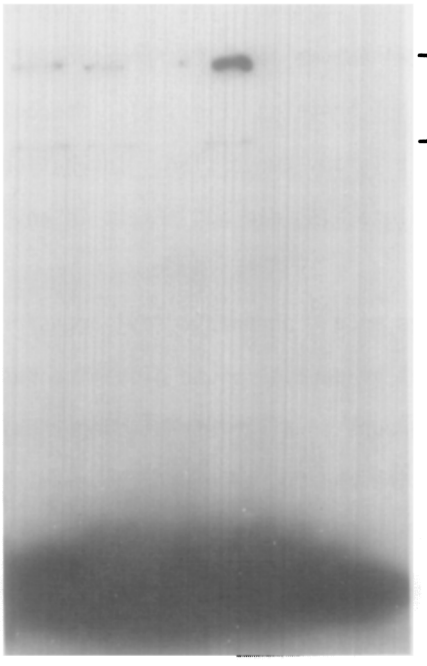

Fig. 2. Inhibition of human placental Factor XIII mediated cross linking of uteroglobin. Crosslinking was done as given in "Materials and Methods" for 1 hr at 37°C with 10 µg uteroglobin alone (lane 5), with 10 µg uteroglobin and 0.3 units placental Factor XIII (lane 4) 10 µg uteroglobin and 0.3 units placental Factor XIII in the presence of 52 nmoles dansylcadavarine (lane 1), 60 nmoles histamine (lane 2) or 1 nmole polylysine (lane 3). Unincubated labelled uteroglobin was also electrophoresed (lane 6).

not [^{125}I] myoglobin (Fig. 4) resulted in the appearance of [^{125}I] label at higher molecular weight regions. The pattern was different from that seen when Factor XIIIa and uteroglobin alone were incubated (Fig. 1). This is likely to be due to the generation of acyl donors by the surface protein interaction with Factor XIIIa which were then transferred to uteroglobin acyl acceptor sites.

Discussion:

The relative abundance of lysine residues in uteroglobin indicates that this protein may indeed be crosslinked by transglutaminase. It is interesting that incubation with Factor XIIIa or the liver enzyme resulted in the incorporation of [^{125}I] uteroglobin predominantly into high molecular weight proteins. This could be due to uteroglobin molecules

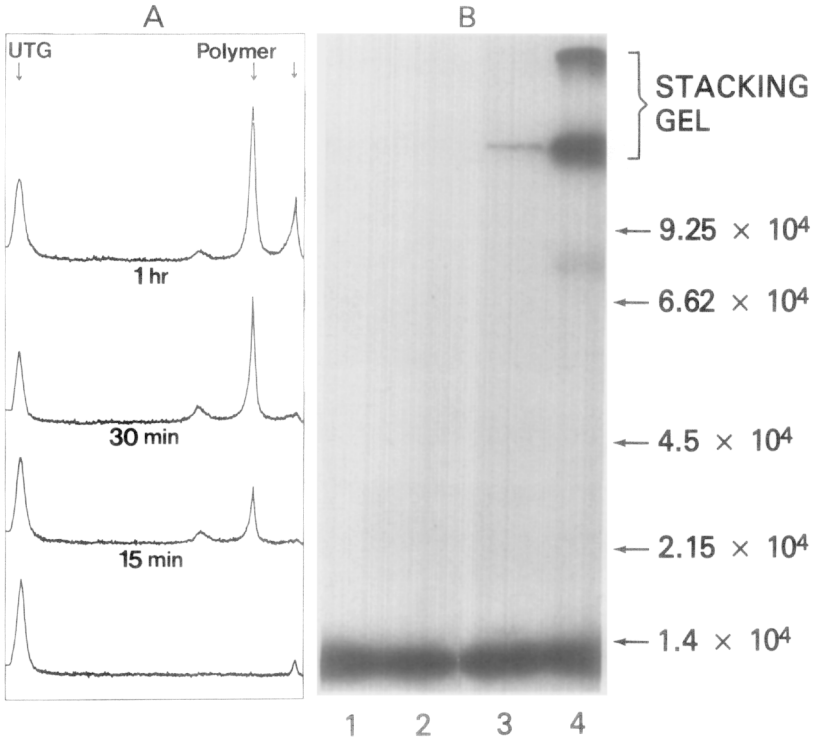

Fig. 3A. Time course of crosslinking between uteroglobin and guinea pig liver transglutaminase. Crosslinking was performed as given in "Materials and Methods" with 0.8 units enzyme and 13 μg uteroglobin for different periods of time as indicated below each tracing. Proteins were separated on a 10% SDS polyacrylamide gel and autoradiographed. A laser densitometric scan of the exposed X-ray film was taken.

3B. Guinea pig liver transglutaminase catalyzed crosslinking of uteroglobin. 0.8 units of guinea pig liver transglutaminase was incubated with 13 μg uteroglobin at 37°C for 1 hr. Crosslinking was performed essentially as given in "Materials and Methods". Proteins were separated on 10% SDS polyacrylamide slab gels and autoradiographed. Lane 1: Uteroglobin incubated without enzyme. Lane 2: Uteroglobin incubated with enzyme in the absence of $CaCl_2$. Lane 3: Uteroglobin and enzyme incubated in the presence of $CaCl_2$ but in the absence of DTT. Lane 4: Uteroglobin and enzyme incubated in the complete reaction mixture. The position of the standard molecular weight markers as visualized by Coomasie blue staining are indicated on the right in daltons.

serving as acyl acceptors of self polymerised Factor XIIIa and not the result of self polymerisation of uteroglobin. Factor XIIIa is known to polymerise in the presence of Ca^{++} (25), as observed by Coomassie Blue staining. The incorporation of MDC and [^{3}H] putrescine into Factor XIIIa also supports this interpretation (data not shown). The fact that uteroglobin could not be labelled with [^{3}H] putrescine or MDC suggests

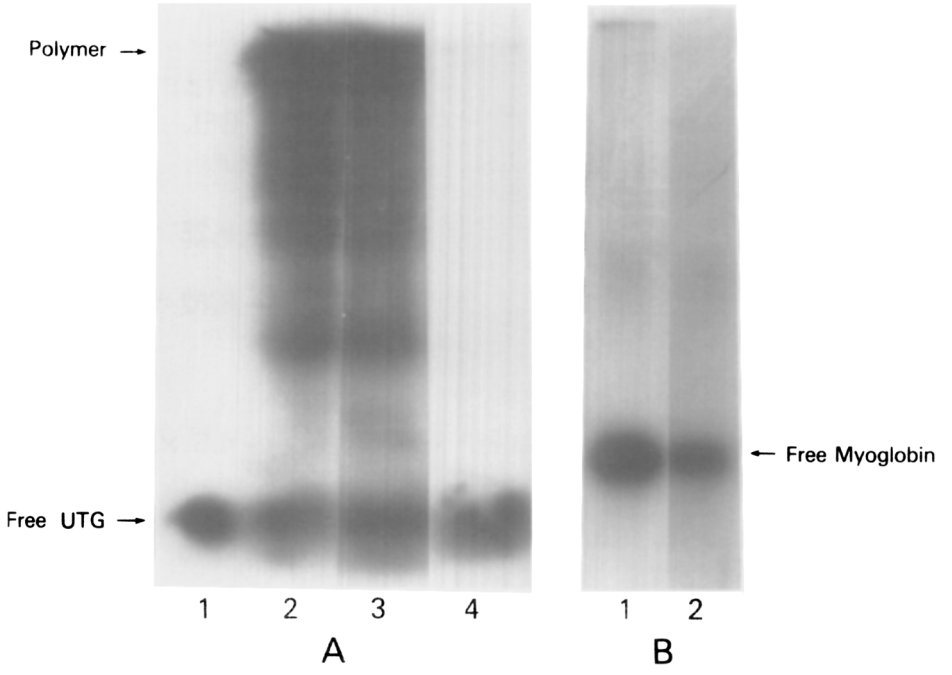

Fig. 4A. Crosslinking of uteroglobin with sperm by human placental Factor XIII. 500 g sperm protein was incubated with 15 µg [^{125}I] uteroglobin in the complete reaction mixture (1 ml) containing 0.2 units (lane 2), 0.1 unit (lane 3) or no placental Factor XIII (lane 4) for 1 hr at 37°C. The sperm were spun down at 800 X g for 10 mins, washed twice with PBS and dissolved in 250 µl SDS sample buffer. Equal aliquots of this were electrophoresed and autoradiographed as given in "Materials and Methods". The sperms were incubated with [^{125}I] uteroglobin in buffer for 10 mins before being added to the enzyme also preincubated for 10 mins in the complete reaction mixture. Unincubated uteroglobin was also run (lane 1).

4B. Absence of crosslinking of myoglobin with sperm by human placental Factor XIII. Crosslinking was done as given above by incubating Factor XIII (0.1 unit) for 1 hr at 37°C with 10 g [^{125}I] myoglobin alone (lane 2) or with 10 µg [^{125}I] myoglobin and 500 µg sperm protein.

that the substrate sites are on uteroglobin lysine residues, not glutamine. Inhibition by polylysine (Fig. 3) supports this observation.

Crosslinking through acyl acceptor involving lysine peptide residues is a unique physiologically regulated process. Fibrin α-chain is also an acyl acceptor for fibronectin (26, 27) and α_2-plasmin inhibitor (28, 29). The fact that HLA-A and B (9) were shown to be acyl donors for transglutaminase and the present finding of covalent attachment of uteroglobin to epididymal cell surface protein is an indication that other cellular proteins could participate in this reaction. We suggest that transglutaminase catalysed

modification of uteroglobin or homologous proteins (30) in mammalian species other than rabbit, may be involved in suppressing the immunogenicity of male gametes reported earlier (17).

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