Biochemical Evidence for a Cytoplasmic 1α,25-Dihydroxyvitamin D₃ Receptor-like Protein in Rat Yolk Sac*

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The yolk sac in rats is an organ of exchanges between the mother and fetus. A vitamin D-dependent calcium-binding protein (CaBP) has been recently described in this organ. This led us to investigate the presence of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) receptor-like proteins in the yolk sac cytosol. For this purpose we have utilized sucrose gradient centrifugation, Scatchard analysis, and DNA-cellulose chromatography. Our results show that cytosol prepared from rat yolk sacs contains a 3.3 S binding protein for 1,25-(OH)₂D₃. The binding is a highly specific, saturable process with high affinity (2 × 10⁻¹⁰ M at 25 °C). The sterol-protein complex binds to DNA-cellulose. The 1,25-(OH)₂D₃ binding protein is present in the yolk sac from at least the 15th day until the 21st day of gestation. In contrast, a binding protein is not found in the amnion, the other component of fetal membranes. The biochemical parameters of the 1,25-(OH)₂D₃ binding protein in the yolk sac are similar to those of 1,25-(OH)₂D₃ cytosolic receptor described in vitamin D target organs. This strongly suggests that the 3.3 S protein in the yolk sac may function as a specific receptor, indicating that this organ may be a new target organ for vitamin D.

There is evidence that 1,25-dihydroxyvitamin D₃, the hormonal form of vitamin D₃, functions at the cellular level in a manner similar to that of other steroid hormones. In the intestine, one of its major target organs, 1,25-(OH)₂D₃ first associates with a specific 3.5 S cytosolic receptor whose characteristics have been extensively studied (1-4). The 1,25-(OH)₂D₃ receptor complex is then translocated to the nucleus (1, 5) and induces the synthesis of mRNAs which code for a vitamin D-dependent calcium-binding protein (CaBP) (6). Intestinal CaBP is thought to be involved in calcium transport (7).

There is also evidence that vitamin D and its metabolites are important during the gestation (8-10). One of their functions may be the regulation of calcium transport across the placenta and the yolk sac which are the organs of exchange between mother and fetuses in rats (11, 12). This possible role is supported by the presence of a CaBP, similar to the intestinal vitamin D-dependent CaBP, in placenta and yolk sac (13, 14). Demonstration of 1,25-(OH)₂D₃ cytosolic receptors, which must be involved in CaBP synthesis, would strengthen the vitamin D sensitivity of these organs. The physiological importance of the rat yolk sac, a nutritive organ which is also an embryonic precursor of the intestine (15), prompted us to investigate the presence of 1,25-(OH)₂D₃ cytosolic receptors in this tissue.

EXPERIMENTAL PROCEDURES

Animals and Materials—Normal pregnant Wistar rats were obtained from Lesieux (France) and fed ad libitum with a normal diet (UAR 103). Male Wistar rats were obtained as weanlings from mothers kept on a vitamin D-deficient diet during 3 months. Tritiated 25-hydroxy[23,24-³H]vitamin D₃ (100 Ci/mmol) and 1,25-dihydroxy[23,24-³H]vitamin D₃ (90 Ci/mmol) were obtained from Amersham (France). Unlabeled 25-OH D₃ was a gift from Roussel Uclaf (France) while 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃) and 1,25-(OH)₂D₃ were kindly obtained from Hoffman La Roche (Switzerland).

Tissue Preparation—Unless otherwise stated, all experiments were performed on rats which were in their 18th day of gestation. Materials were obtained under light ether anaesthesia via cesarean section. The yolk sacs were dissected free from amnion, fetus, and placenta and rinsed thoroughly in ice-cold Tris buffer (0.01 M Tris-HCl, pH 7.4, 0.5 M KCl, 0.0015 M EDTA, 0.001 M dithiothreitol, 0.01 M sodium molybdate). All subsequent operations were made in a cold room at 4 °C. The yolk sacs were cut into small pieces and homogenized in 3 volumes of cold Tris buffer containing 0.23 unit/ml of protease inhibitor (Aprotinin, from Sigma) by 3 passes in a Potter Elvehjem. The homogenate was centrifuged at 9,000 g for 15 min in a Sorvall RCB centrifuge and the resulting supernatant was recentrifuged at 120,000 g for 60 min in a Beckman L2-75 ultracentrifuge. The final high speed supernatant without the fluffy lipid layer was taken as cytosol. In some experiments ammonium sulfate precipitations of cytosolic material were carried out by addition of solid (NH₄)₂SO₄ (250 mg/ml of cytosol). After 20 min of gentle stirring, the turbid solution was centrifuged at 8,000 g for 15 min. The pellet was then redissolved in Tris-buffer. Incubations were always performed without delay since we observed a decrease of the 3.3 S binding activity after freezing.

In one set of experiments, yolk sacs were incubated intact in 1 ml of Eagle’s minimal essential medium containing 5 × 10⁻¹⁰ M 1,25-(OH)₂[³H]D₃ (in 50 μl of ethanol), at 4 °C overnight. Intact yolk sacs were washed repeatedly with Tris buffer. Cytosol was then prepared and aliquots (0.2 ml) were directly submitted to sucrose gradient sedimentation analysis. Fetal proximal small intestines and fetal kidneys removed from pregnant rats on the last day of gestation were incubated and treated in the same manner.

Sucrose Density Gradients—Linear rat to 30% (w/w) sucrose density gradients (4 ml) were prepared in Tris buffer and allowed to equilibrate at 4 °C for 1 h. The cytosol or its resolubilized (NH₄)₂SO₄ precipitate were incubated with a 1 μM concentration of the appropriate labeled sterol at 4 °C for 3 h. Aliquots (1 to 2 mg of protein in 0.2 ml) were sedimented at 4 °C for 17 h at 240,000 x g in a Beckman SW 60 Ti rotor. [³C]Ovalbumin and [³C]Bovine serum albumin, obtained from New England Nuclear (France), were sedimented in parallel gradients as markers. 35 fractions were collected from the top.
and radioactivity measured in 10 ml of Picofluor (Packard Instruments) using a SL 40 Intertechnique counter.

Yolk Sac Cellulose Chromatography—Yolk sac cytosols were incubated with 1 nm 1,25-(OH)2[3H]D3 in presence of 10 nM unlabeled 25-OHD3 at 4 °C for 1 h and 30 min and then precipitated by (NH4)2SO4. The pellet was resuspended in 4 ml of buffer (0.01 M Tris-HCl at pH 7.4, 0.001 M EDTA, 0.012 M polyethylene glycol) and applied to a column (1 × 6 cm) of DNA-cellulose (Sigma). The column was first eluted with 15 ml of the same buffer, and then with 30 ml of buffer with 0.15 M KCl, and finally with 60 ml of a linear gradient from 0.15 to 0.6 M KCl in buffer. One-mI fractions were collected and counted for radioactivity.

Binding Studies—Cytosol was prepared using yolk sacs pooled from 8 pregnant rats. In polyallomer test tubes, 0.5-ml aliquots of resolubilized precipitated cytosol (about 1 mg of protein/tube) were incubated in duplicate or triplicate with steroid (in 30 μl of ethanol) in a shaking-water bath with 25 °C for 40 min. Bound steroid was precipitated by addition of 1 ml of 35% polyethylene glycol in Tris buffer. The precipitate was collected by centrifugation at 9000 × g for 1 h and removed by cutting the bottom of the tube. The precipitate was extracted with methanol and the solution was counted for 3H (16).

For Scatchard analysis (17), incubations were performed with increasing concentrations of 1,25-(OH)2[3H]D3 in the absence or presence of a 200-fold molar excess of unlabeled 1,25-(OH)2D3. Specifically bound steroid was calculated by subtracting the nonspecific binding from the total binding.

Other Assays—Protein concentrations were measured using a method modified from Lowry (18). Cytosolic calcium-binding protein was quantified by a specific radioimmunoassay (19).

RESULTS AND DISCUSSION

Sucrose Gradient Analysis—Cytosol from rat yolk sac was examined for the presence of a 1,25-(OH)2D3 specific binding protein by sucrose density gradient analysis. When cytosol was incubated with 1,25-(OH)2[3H]D3, the radioactivity was associated with two peaks, sedimenting at approximately 3.3 S and 5.5 S (Fig. 1A). In contrast, 25-OH[3H]D3 was bound only to the 5.5 S peak (Fig. 1A). The two binding activities of the yolk sac cytosol could be distinguished according to their specificity: the addition of an excess of 1,25-(OH)2D3 induced the disappearance of both peaks while unlabeled 25-OH-D3 favored the 3.3 S 1,25-(OH)2[3H]D3 binding by greatly reducing the 5.5 S peak (Fig. 1B). This indicates that the 3.3 S macromolecules bind only 1,25-(OH)2D3 while the 5.5 S ones are able to bind both metabolites. The binding activities and specificities we have observed in the rat yolk sac were identical with those of duodenal mucosa from vitamin D-deficient rats taken as a control (Fig. 1D). Moreover, the results are similar to those reported for the main vitamin D target organs (1—4, 20—25), where the 3.3 S peak is the 1,25-(OH)2D3 receptor. The 5.5 S activity has been reported as an artifactual complexed form of the serum vitamin D binding protein generated during the cytosol preparations of most nucleated tissues (26, 27). To minimize the 5.5 S contamination, cytosol was precipitated by ammonium sulfate before 1,25-(OH)2[3H]D3 incubation (20). Fig. 1C shows the enrichment of the 3.3 S activity in the resolubilized ammonium sulfate precipitate of the yolk sac cytosol. Therefore, this partial purification has been used for the further characterization of the 3.3 S binding activity.

It is interesting to note that the 3.3 S cytosolic binding activity was also detected by sedimentation analysis when the intact yolk sac was incubated with 1,25-(OH)2[3H]D3 prior to homogenization (Fig. 2A). This method gave a preferential association of 1,25-(OH)2[3H]D3 to the 3.3 S peak due to a substantial reduction of the 5.5 S binding activity. When the same procedure was applied to kidney and proximal small intestine from rat fetuses on the last day of gestation, the cytosol from these fetal organs also displayed a 3.3 S specific 1,25-(OH)2[3H]D3 binding activity (Fig. 2B). These results suggest that kidney and duodenum even in the fetus, possess the 3.3 S 1,25-(OH)2D3 receptor characteristic of these organs in the adult.

Treatment of the resolubilized ammonium sulfate precipitate of the yolk sac cytosol with trypsin or N-ethylmaleimide prior incubation with 1,25-(OH)2[3H]D3 provoked a marked decrease of the 1,25-(OH)2[3H]D3 binding to the 3.3 S macromolecules while the 5.5 S complex was less affected (Fig. 3). These results indicate that the 3.3 S peak is a protein and that sulfhydryl groups are implicated in the recognition of the hormone. Degrading agents (temperature, trypsin, N-ethylmaleimide) were much more effective on the apoprotein than on the 1,25-(OH)2[3H]D3 3.3 S protein complex (Fig. 3). This illustrates the protective influence of the hormone upon the degradation of the 3.3 S protein.

DNA-Cellulose Chromatography—Yolk sac cytosol was found to contain a significant amount of 1,25-(OH)2D3 binding macromolecules which absorbed to DNA-cellulose and eluted in the region of 0.3 M KCl (Fig. 4). Radioactivity was never detected in this region when incubations were performed with 25-OH[3H]D3 or with 1,25-(OH)2[3H]D3 in presence of a 400-
fold molar excess of unlabeled 1,25-(OH)2D3. Such a binding to DNA-cellulose has been shown to be a property of steroid hormone receptors (28), including 1,25-(OH)2D3 receptors (20, 29-31).

**Competition**—The reactivity of several vitamin D metabolites and steroids in competing for the binding of 1,25-(OH)2D3 to yolk sac resolubilized precipitate was studied. As shown in Fig. 5, 1,25-(OH)2D3 was the best competitor. At 50% inhibition, 1,24,25-(OH)3D3 was about 10 times less potent than 1,25-(OH)2D3 while 25-OHD3 and 24,25-(OH)2D3 relative potencies were much lower. Other steroids (cortisol, progesterone, oestradiol, testosterone) even at a 10,000 molar ratio did not compete at all with 1,25-(OH)2D3. Thus, in the rat yolk sac, the specificity of binding is comparable to that described for the 1,25-(OH)2D3 receptor in rat intestine (3), bone (23), and kidney (20).

**Scatchard Analysis**—The saturation analysis performed at 25 °C showed that the specific binding was a saturable process (Fig. 6). Scatchard treatment of the specific binding data yielded an apparent Kd of 2 x 10^-10 M for 1,25-(OH)2D3. Such a value is in the range of those reported in vitamin D target organs (3, 4, 20, 23).

In the course of the last third of pregnancy, we have detected by sucrose gradients the 3.3 S activity in the yolk sac cytosol as early as the 15th day and until the end of gestation. By contrast, the 3.3 S specific binding protein was never
observed in the cytosol prepared from the amnion, the other main component of the fetal membranes. This tissue specificity can be related to the respective concentrations of CaBP: while CaBP concentration was always about 3 μg/mg of cytosolic protein in the yolk sac, its value was negligible in the amnion (about 0.04 μg/mg of cytosolic protein).

Our data clearly indicate that, as early as day 15 of gestation, the rat yolk sac contains a highly specific cytosolic binding protein for 1,25-(OH)2D3. Its biochemical characteristics are those required for steroid hormone receptors. In addition, they are the same as those of 1,25-(OH)2D3 receptors in other vitamin D target organs (3, 4, 20, 23, 29, 30). The presence of specific receptors for 1,25-(OH)2D3 together with an hormonal molecular response, CaBP synthesis, points out the sensitivity of the yolk sac tissue to 1,25-(OH)2D3. Thus, through the 1,25-(OH)2D3 receptors in rat yolk sac and in placenta (31, 32), vitamin D may participate in the calcium homeostasis within the fetoplacental unit. As another consequence of the present work, the rat yolk sac must now be considered as a hormone-responsive organ. Furthermore, this study in addition to our previous report on vitamin D-dependent CaBP in yolk sac and fetal intestine (14) re-emphasizes the degree of relationship existing between the endodermal cells of the yolk sac and the epithelial columnar cells of the fetal or neonatal intestine (11, 15).

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