Evidence for two structurally related progesterone receptors in chick oviduct cytosol

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Received 19 April 1983

The existence of two progesterone receptor forms present in crude cytosol of chick oviduct has been demonstrated by photoaffinity labelling using [^{3}H]R5020. On SDS–polyacrylamide gels these two forms exhibit app. M_r-values of 79000 and 109000 corresponding to the progesterone receptor forms A and B. Peptide maps of photoaffinity-labelled steroid receptors have been established by limited proteolysis with α-chymotrypsin. The peptide map obtained for chick oviduct cytosol progesterone receptor crosslinked with [^{3}H]R5020 proved to be the sum of peptides obtained from partially purified preparations of forms A and B. The peptide maps of both progesterone receptor forms were identical for peptides below the M_r-value of form A, indicating extensive homology of the two forms. A significantly different peptide pattern was observed for the rat liver glucocorticoid receptor crosslinked with [^{3}H]triamcinolone acetonide. Prolonged proteolysis with chymotrypsin gave rise to peptides with M_r-values of 6000 and 10000 from the hormone-binding domain of progesterone and glucocorticoid receptors, respectively.

Steroid hormone receptor *Peptide mapping* *Photoaffinity labelling* *Hormone binding domain*

1. INTRODUCTION

Multiple forms of the chick oviduct progesterone receptor have been described [1–4], at least two of which appear to be the product of proteolytic degradation [5]. A 6 S progesterone receptor was resolved into components 'A' and 'B' by DEAE-cellulose chromatography and it was found that only A bound to DNA [6]. Different characteristics of both forms with respect to their interaction with ion-exchange resins has facilitated their separation and isolation and on SDS–polyacrylamide gels app. M_r-values of 79000 and 117000 have been determined for the A and B form, respectively (review [1]). Recently, using affinity chromatography, the isolation of a molybdate stabilized chick oviduct progesterone receptor has been described, consisting of either 2 bands (90 kDa and 104 kDa) on SDS gels [4] or a single 85-kDa polypeptide chain [3]. Both of these reports appear to be at variance with the above studies.

Photoaffinity labelling with unmodified ligands containing unsaturated ketone functions has proven to be an important tool in the investigation of steroid hormone-binding proteins [7–11] and chromosomal binding sites of ecdysterone [12,13]. If this crosslink occurred exclusively in the hormone-binding domain it should be possible to establish peptide maps of receptors by limited proteolysis even in crude preparations. As such peptide maps are highly specific for a given protein [14], in combination with competition studies they would unequivocally prove the isolation of a specific hormone receptor.

Here, we have tried to establish peptide maps of the chick oviduct progesterone receptor throughout a purification procedure. The aim was to define those receptor forms which can be detected in crude cytosol and to clarify the rela-

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Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

tionship between these forms on the basis of the structural information provided by the peptide maps.

Furthermore, the applicability of this kind of analysis to other steroid receptor systems is reported, as well as the possibility to obtain defined peptide fragments from the hormone-binding domain of steroid hormone receptors.

2. MATERIALS AND METHODS

2.1. *Purification of progesterone receptor*

The partially purified chick oviduct progesterone receptor used in this study is obtained as an intermediate in a purification procedure derived by modifications of standard techniques [1] which will be detailed elsewhere (in preparation). Briefly, cytosol from diethylstilbestrol (DES)-stimulated chicks or laying hens was labelled using [^{3}H]R5020 ([17α-*methyl*-^{3}H]-17,21-dimethyl-19-nor-pregn-4,9-diene-3,20-diene) (NEN) in the presence of 10^{-6} M cortisol and 10^{-7} M dexamethasone (non-specific binding of R5020 was $\leqslant$10%) and subjected to a serial arrangement of chromatographic steps. Final purification for the progesterone receptor was about 770-fold for form A and 1000-fold for form B. Photoaffinity labelling (described below) was done at various stages of the purification. In cases where crude cytosol was to be irradiated, samples after labelling were treated with 1 vol. wet phosphocellulose (P11, Whatman) for 5 min followed by dextran-coated charcoal (0.05%/0.5%) adsorption for 30 min on ice in order to remove coloured proteins and free ligand, respectively.

2.2. *Partial purification of glucocorticoid receptor*

The 90-kDa [15] (fresh tissue) and the 40-kDa [16] (frozen tissue) form of rat liver glucocorticoid receptor labelled with [^{3}H]triamcinolone acetonide (NEN) were purified about 150-fold according to standard procedures and crosslinked.

2.3. *Photoaffinity labelling*

Crosslinking of receptor preparations was performed using a 1-kW Mercury-Xenon lamp (Oriel) and a 2 mm thick long-pass filter (WG320, Schott) in a jacketed cylindrical quartz cuvette, cooled to -10°C. The solution was mixed with a magnetic stirrer during irradiation (12 min for cytosolic preparations and 1–5 min for purified preparations). Crosslinking efficiencies varied from 5–12% of specifically bound hormone (measured with the dextran-charcoal assay [17]) as determined by trichloroacetic acid precipitation (final conc. 10%).

2.4. *Peptide mapping*

Peptide maps were generated by limited proteolysis with α-chymotrypsin (Worthington, 3 × crystallized, 45–70 units/mg). A stock solution was prepared containing 50 mg/ml chymotrypsin and 1 mg/ml Nα-4-tosyl-L-lysine chloromethylketone (TLCK) in 100 mM Na-phosphate (pH 7.2), 1 mM Na_2EDTA (digest buffer) and aliquots were frozen at -20°C. Receptor preparations after photoaffinity labelling were precipitated at 40% ammonium sulphate or by 10% trichloroacetic acid (40 kDa glucocorticoid receptor preparations) and the protein pellets were solubilized in digest buffer containing 8 M freshly deionized urea. After dilution with digest buffer to 2 M urea, digestions of aliquots were performed at 37°C for 30 min by varying the enzyme to protein ratio (E/P) as indicated in the figures. For complete digestion, crosslinked receptor preparations were incubated with an excess of TLCK-enzyme (E/P = 3) for 1 h at 37°C.

2.5. *Gel electrophoresis and fluorography*

SDS–polyacrylamide gel electrophoresis of digested samples was performed on gradient gels as indicated in the figure legends using the high-M_r [^{14}C]protein marker kit from BRL as standard. Fluorography was carried out with EN^{3}HANCE (NEN) according to the manufacturer's instructions. After complete digestions, receptor preparations were analyzed in 15% polyacrylamide gels containing 100 mM Na-phosphate (pH 7.2), 0.1% SDS, and 6 M urea as recommended by BRL (progesterone receptor) or in 8.75–15% SDS–polyacrylamide gels containing a 0–8 M gradient of urea (glucocorticoid receptor) using prestained low-M_r proteins (BRL) as reference. The gels were extensively washed in fixing solution and cut into 2-mm slices which were dissolved in H_2O_2 overnight at 55°C. After addition of scintillation fluid (D22, Roth), the vials were stored for 24 h in the dark at 4°C and the radioactivity was measured in a liquid scintillation counter.

3. RESULTS

3.1. *Progesterone receptor*

When progesterone receptor in crude cytosol of DES-stimulated chicks or laying hens is crosslinked with [^{3}H]R5020, only two radioactive-labelled bands are visualized on the fluorograph of SDS gels (fig.1A, first lane), app. M_r-values of about 79000 and 109000. After partial purification of progesterone receptor according to protocols used to separate forms A and B [1], only one of these proteins can be detected after photoaffinity labelling. For example, after elution of form A from DEAE–Sepharose with 150 mM NaCl only the 79-kDa protein can be crosslinked (fig.1B, first lane). The same result is obtained when progesterone receptor form A was 770-fold purified (not shown). After a 1000-fold purification of form B, only the 109-kDa protein can be photoaffinity-labelled with R5020 (fig.1C, first lane). These results strongly suggest that the cytosol of chick oviduct contains only two functional forms of the progesterone receptor, having the characteristics of the forms A and B in [1]. It should be noted that no difference in results was found when the homogenization of oviducts was performed in the presence or in the absence of molybdate (not shown).

Fig.1A–C shows the peptide maps obtained by limited proteolysis with TLCK–α-chymotrypsin of photoaffinity-labelled chick oviduct progesterone receptor at various stages of purification. The map obtained from crude cytosol preparations (fig.1A) evidently represents the sum of the peptides derived from partially purified forms A (fig.1B) and B (fig.1C). The peptides visualized by this technique are generated by chymotryptic digestion at various distances from the point where the radioactive ligand has been crosslinked to the receptor molecule. The appearance of distinct peptide bands indicates that the steroid is exclusively crosslinked to a specific region of the receptor protein, namely the hormone-binding domain. Differences in the relative intensities of the peptide bands obtained are probably due to different rate constants of hydrolysis, as chymotryptic attack is influenced by the nature of the amino acid residue adjacent to the susceptible bond [18]. An influence

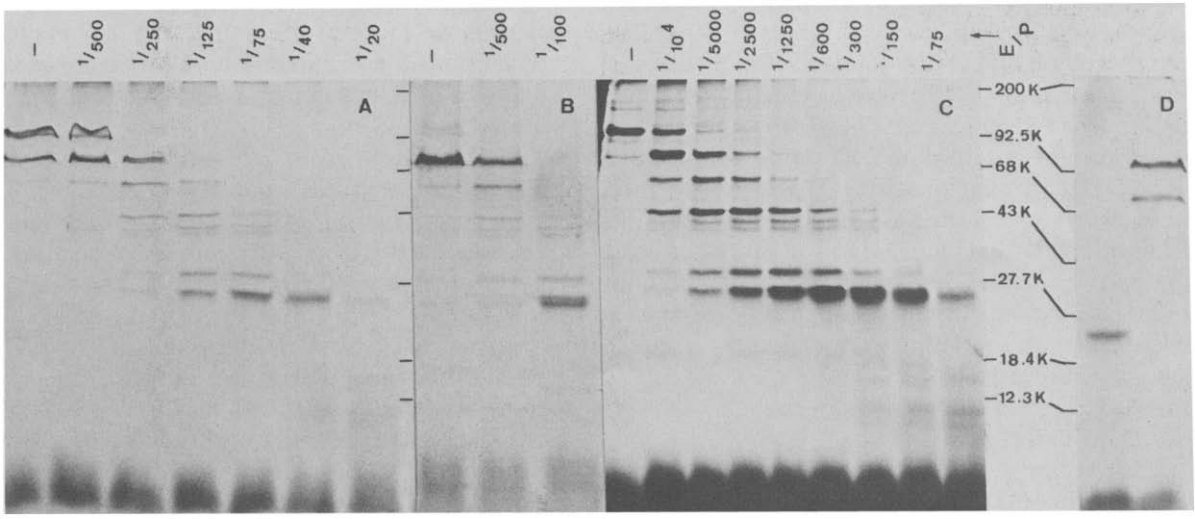

Fig.1. Fluorography of peptide maps of chick oviduct progesterone receptor obtained by limited proteolysis with TLCK–α-chymotrypsin at various purification levels. (A) Form A and B from phosphocellulose treated cytosol, labelled with [^{3}H]R5020 (20 nM) and crosslinked as in section 2. Aliquots were digested with increasing enzyme to protein ratios (E/P) as indicated on top. (Exposure time was equivalent to 1.4×10^5 cpm crosslinked × days.) (B) Peptide map of form A obtained after separation from form B on DEAE–Sepharose (exposure time: 1.8×10^5: cpm × days). (C) Peptide map of form B 1000-fold purified (section 2). Exposure time: 3.9×10^5 cpm × days. (D) Fluorography of cytosolic progesterone receptor preparation crosslinked before (right) and after (left) incubation with 3 mM Ca^{2+} at 0°C for 10 min; (A–C) 8.75–15%; (D) 7.5–12% SDS–polyacrylamide gel containing 0–8 M urea.

of the ternary structures of the receptor protein may be discounted as the protein was denatured before addition of the enzyme and digestion was performed in 2 M urea. The radioactivity visible at the dye front is due to free hormone which migrates in SDS–micelles and to end-products of the digestion which are not resolved by this gel system. These peptide maps are consistent throughout the purification procedure with respect to the localization and relative intensities of labelled peptide bands.

A very surprising observation was that the peptide maps generated from the separated forms A and B of the progesterone receptor are identical in terms of peptides below the M_r of form A. This indicates an extensive sequence homology if not the partial identity of the two receptor forms. Indeed, progesterone receptor form B is initially cleaved to give a peptide of slightly higher M_r than undigested form A (see the doublet band in fig.1A, E/P = 1/250) and is then processed further in exactly the same way as form A.

Ca^{2+} treatment of chick oviduct cytosol is known to convert the progesterone receptor into a smaller 'mero'-receptor, probably generated by Ca^{2+}-activated proteolytic cleavage [5]. In this study, oviduct cytosol was subjected to a 10 min treatment with 3 mM Ca^{2+} on ice before photoactivation and electrophoresis on an SDS–polyacrylamide gel. Only one radioactive peptide can be observed on the fluorograph of this gel (fig.1D), having the same M_r as one of the predominant peptide bands seen after chymotryptic digest (about 23000). This indicates that an endogenous protease of a similar type, although more specific than chymotrypsin, may be responsible for the Ca^{2+}-activated cleavage of the progesterone receptor.

3.2. *Glucocorticoid receptor*

A variety of steroid hormone-binding proteins can be photoaffinity labelled with unmodified ligands [7–11] and therefore offer the possibility to establish peptide maps in the same way as described above for the progesterone receptor. Fig.2 shows a peptide map of the 40 kDa form of the glucocorticoid receptor prepared from frozen rat liver crosslinked with [^{3}H]triamcinolone acetonide. As expected, a pattern significantly different from that of the chick oviduct progesterone receptor is visualized, demonstrating that the technique is specific for a given steroid hormone receptor and that each receptor can be easily identified.

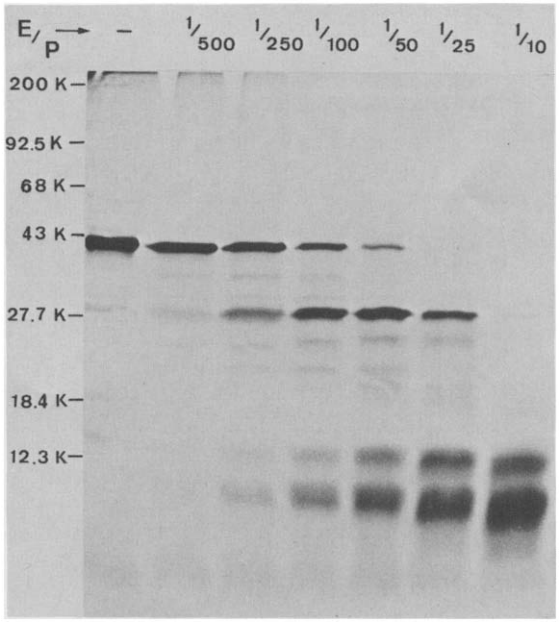

Fig.2. Peptide map of an about 150-fold purified 40-kDa glucocorticoid receptor from frozen rat liver (section 2); 8.75–15% SDS–polyacrylamide gel. (Exposure time: 2.4×10^5; cpm × days.)

3.3. *Complete digest of progesterone and glucocorticoid receptors*

The specificity of the crosslinking reaction permits the identification of a labelled peptide from the hormone-binding domain after complete chymotryptic digest. To determine the size of this fragment, two gel systems were chosen that permit the identification of peptides down to an M_r of about 2000. In order to eliminate non-crosslinked hormone, the gels were extensively washed in the fixing solution. Fig.3 shows the distribution of radioactivity on a 15% SDS–urea–polyacrylamide gel, for an extensive chymotryptic digest of progesterone receptors A and B from chick oviduct cytosol. Only one major peak corresponding to an M_r of about 6000 is obtained. The same result was observed when form A and B were separated prior to digestion (not shown). When the 90-kDa form of crosslinked rat liver glucocorticoid receptor prepared from fresh tissue is chymotrypsinized,

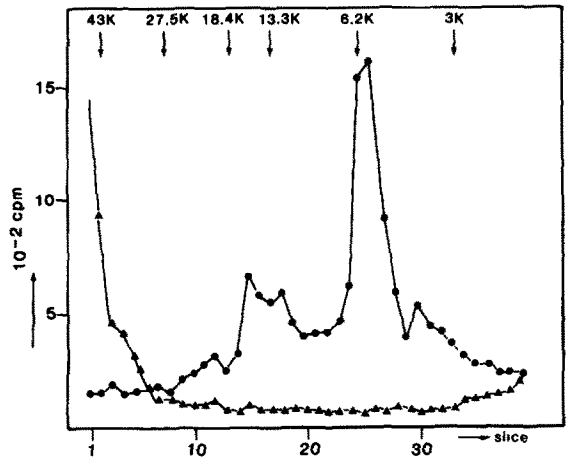

Fig.3. Distribution of radioactivity on a 15% SDS–urea gel (section 2) after complete digest of crosslinked chick oviduct progesterone receptor A and B from cytosol with TLCK–chymotrypsin: (▲) before, (●) after digestion with TLCK-chymotrypsin for 1 h at 37°C (E/P = 3).

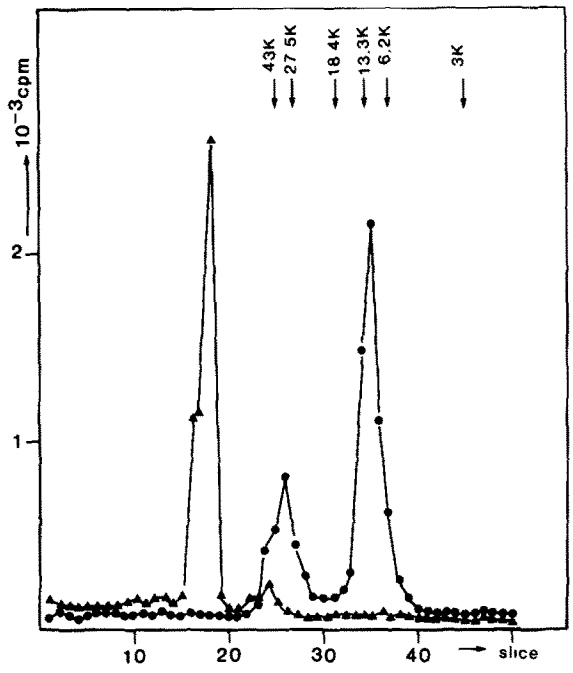

Fig.4. Distribution of radioactivity on 8.75–15% SDS–polyacrylamide gel containing an 0–8 M urea gradient after crosslinking 150-fold purified 90 kDa glucocorticoid receptor from unfrozen rat liver: (▲) before, (●) after digestion of crosslinked receptor with TLCK–chymotrypsin for 1 h at 37°C (E/P = 3).

the hormone-binding domain is digested to a fragment with an M_r of about 10000 (fig.4). A peptide of this size is also seen on extensive digestion of the 40 kDa form (fig.2, E/P = 1/10). The small peak in fig.4 preceding the 10 kDa fragment has a size of about 40 kDa, indicating that the 40 kDa glucocorticoid receptor from frozen tissue is the result of a proteolytic degradation of the 90 kDa form, probably induced by lysosomal breakage during the freezing–thawing cycle [19].

4. DISCUSSION

Peptide mapping by limited proteolysis, established by authors in [14], is a powerful technique for the identification of particular proteins, clearly differentiating even closely related proteins like α- and β-tubulin [14]. This kind of analysis, however, requires the isolation of the protein in considerable amounts. Photoaffinity labelling permits the identification of a protein present at a very low abundancy in cytosolic preparations of high complexity (e.g., chick oviduct progesterone receptor). In conjunction with peptide mapping, photoaffinity labelling permits the unequivocal identification of steroid binding proteins at any stage of a purification procedure, starting even with crude cytosol.

Here, photoaffinity labelling of chick oviduct progesterone receptor revealed two bands at M_r 79000 and 109000 on SDS–polyacrylamide gels. These proteins possess the characteristics of the form A and form B as in [1]. Peptide mapping showed that the partially purified forms A and B are in fact identical with the two functional progesterone receptor forms which can be observed in chick oviduct cytosol.

Recently the purification of chick oviduct progesterone receptor by affinity chromatography has been reported [3,4], displaying different characteristics on SDS–polyacrylamide gels (see section 1) than reported here. By means of peptide mapping as described here, it should be simple to ascertain whether the different purification procedures in fact yield different proteins, or, for example, merely intermediate proteolytic fragments of the same protein.

The glucocorticoid receptor complex contains sites which are preferentially sensitive to proteolytic attack. It has been found that these sites

define functional subunits and 3 domains have been identified described as steroid-binding activity (domain A), DNA-binding activity (domain B) and immunoreactivity (domain C) [19]. The data presented here appear to reflect this structural composition. Whereas the intact receptor (domains A, B and C) is the 90 kDa protein in fig.4, the 40 kDa form (domain A + B) is visualized in fig.2 (left lane) and the strong band at an M_r of about 27700 (fig.4, E/P =1/50) probably represents domain A.

The results obtained by comparative peptide mapping of the progesterone receptors forms A and B indicate extensive structural homology of the two forms. Three possible explanations could account for this fact. The receptor forms A and B may result from gene duplication or distinct splicing events from a single gene. Alternatively, the similarity of the peptide maps may be explained as a consequence of post-translational proteolysis from a unique mRNA. Cloning of the receptor gene(s) will serve to distinguish between these possibilities.

The single chymotryptic peptide obtained from the hormone-binding domain of the progesterone receptor can be easily identified after photoaffinity labelling using radioactive hormone. Thus, the amino acid sequence of this fragment may be determined after further purification. This sequence would be particularly useful for the synthesis of oligodeoxynucleotide probes which would in turn constitute a powerful tool for the identification of the receptor gene(s).

After the completion of this manuscript, a photoaffinity labelling study of the chick oviduct progesterone receptor also using R5020 as ligand appeared in [20]. Although they observed high levels of non-specific labelling and extensive proteolysis of the receptor protein in crude cytosol, which did not allow them to identify form A and B in cytosol preparations, there is, in general, an excellent agreement between their results and ours.

ACKNOWLEDGEMENTS

We would like to express our thanks to A. Alberga for expert criticism and stimulating discussions and to M. Ober for excellent technical assistance. This work was started while H.G. was a recipient of a post-doctoral fellowship from the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Vedeckis, W.V., Schrader, W.T. and O'Malley, B.W. (1978) in: Biochemical Actions of Hormones (Litwack, G. ed) vol.5, pp.321–372, Academic Press, New York.
[2] Sherman, M.R., Ateiza, S.B.P., Shansky, J.R. and Hoffman, L.M. (1974) J. Biol. Chem. 249, 5351–5363.
[3] Renoir, J.-M., Yang, C.-R., Formstecher, P., Lustenberger, P., Wolfson, A., Redeuilh, G., Mester, J., Richard-Foy, H. and Baulieu, E.-E. (1982) Eur. J. Biochem. 127, 71–79.
[4] Puri, R.K., Grandics, P., Dougherty, J.J. and Toft, D.O. (1982) J. Biol. Chem. 257, 10831–10837.
[5] Sherman, M., Pickering, L., Rollwagen, F. and Miller, L. (1978) Fed. Proc. FASEB 37, 167–173.
[6] Schrader, W. and O'Malley, B. (1972) J. Biol. Chem. 247, 51–59.
[7] Taylor, C.A. jr, Smith, H.E. and Danzo, B.J. (1980) Proc. Natl. Acad. Sci. USA 77, 234–238.
[8] Dure IV, L.S., Schrader, W.T. and O'Malley, B.W. (1980) Nature 238, 784–786.
[9] Westphal, H.M., Fleischmann, G. and Beato, M. (1981) Eur. J. Biochem. 119, 101–106.
[10] Nordeen, S.K., Lan, N.C., Showers, M.O. and Baxter, J.D. (1981) J. Biol. Chem. 256, 10503–10508.
[11] Dellweg, H.-G., Hotz, A., Mugele, K. and Gehring, U. (1982) EMBO J. 1, 285–289.
[12] Gronemeyer, H. and Pongs, O. (1980) Proc. Natl. Acad. Sci. USA 72, 2108–2112.
[13] Gronemeyer, H., Hameister, H. and Pongs, O. (1981) Chromosoma (Berlin) 82, 543–559.
[14] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[15] Wrange, Ö., Carlstedt-Duke, J. and Gustafsson, J.-A. (1979) J. Biol. Chem. 245, 9284–9290.
[16] Westphal, H.M. and Beato, M. (1980) Eur. J. Biochem. 106, 395–403.
[17] Milgrom, E. and Baulieu, E.E. (1969) Biochim. Biophys. Acta 194, 602–605.
[18] Smyth, D.G. (1967) Methods Enzymol. 11, 214–231.
[19] Carlstedt-Duke, J., Okret, S., Wrange, Ö, and Gustafsson, J.-A. (1982) Proc. Natl. Acad. Sci. USA 79, 4260–4264.
[20] Birnbaumer, M., Schrader, W.T. and O'Malley, B.W. (1983) J. Biol. Chem. 255, 1637–1644.