J. Steroid Biochem. Molec. Biol. Vol. 38, No. 4, pp. 407–413, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

IN VITRO PHOSPHORYLATION AND HORMONE BINDING ACTIVATION OF THE SYNTHETIC WILD TYPE HUMAN ESTRADIOL RECEPTOR

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(Received 10 August 1990)

Summary—A tyrosine kinase purified from calf uterus activates the hormone binding of endogenous estradiol receptor (ER) predephosphorylated and preinactivated by a nuclear phosphotyrosine phosphatase. The kinase also activates and phosphorylates the human estradiol receptor HEO synthesized *in vitro*, which differs from the wild type receptor HEGO because a glycine is replaced by a valine at position 400. Moreover, the kinase activates and phosphorylates a deletion mutant of HEO which consists almost exclusively of the hormone binding domain.

Using HEGO and HEO in parallel and measuring both binding activation and phosphorylation of ER we now observe that the wild type receptor is a good kinase substrate, slightly better than HEO. Furthermore, HEGO like the calf uterus receptor in the presence of estradiol, stimulates the kinase. From present findings it appears that ER and uterus tyrosine kinase are functionally associated and that this association is abolished by glycine to valine substitution at position 400 of ER.

INTRODUCTION

The uterus estradiol receptor (ER) is phosphorylated on tyrosine *in vitro* [1] as well as *in vivo* [2]. This receptor is a substrate of a uterus nuclear phosphotyrosine phosphatase which abolishes its hormone binding [3, 4] and a uterus cytosol tyrosine kinase which restores this binding [1, 5]. The kinase is stimulated by estradiol–receptor complex [6].

The human ER cDNA clone POR8 has been isolated from a cDNA library of the human breast cancer cell line MCF-7 [7, 8]. The receptor synthesized *in vitro* from this cDNA (HEO) binds hormone with high affinity, but low efficiency. Only a small percentage of the synthetic receptor binds hormone [9]. The uterus kinase activates most of the non-hormone binding HEO receptor. This activation requires phosphorylation on tyrosine of the hormone binding domain [10]. It has recently been observed that the ER cDNA clone POR8 contains an artefactual point mutation which results in the presence of a valine instead of a glycine at position 400 [11]. This mutation alters the hormone binding properties of the receptor to some extent.

The wild type human ER (HEGO) with the glycine at position 400, binds hormone with low efficiency, like HEO. Therefore, we have used this receptor as a substrate of the uterus kinase to verify whether the wild type receptor also acquires hormone binding through phosphorylation by the uterus kinase. The results obtained with the wild type HEGO confirm those reported for the point mutated HEO [9]. However interesting differences have been observed: the tyrosine kinase activates and phosphorylates the wild type HEGO a little more efficiently than the point mutated HEO receptor showing that HEGO is an excellent substrate for the kinase. More importantly, while addition of exogenous estrogen–receptor complex is needed to significantly activate the kinase in the experiments performed using HEO as a substrate [10], this addition is not necessary in the case of experiments with HEGO. The synthetic wild type receptor like the uterus receptor in complex with estradiol [6] is able to activate the kinase. Therefore it appears that glycine-valine mutation at position 400 present in HEO abolishes the prop-

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erty of the receptor to activate the kinase but does not substantially modify the ability of the receptor to act as a substrate for this enzyme.

These findings raise the intriguing possibility that mutations on the estradiol receptor structure could interfere with the estradiol responsiveness of tissues, by impairing the tyrosine phosphorylation process.

MATERIALS AND METHODS

Materials

[^{35}S]methionine (800 Ci/mmol), [γ-^{32}P]ATP (10 Ci/mmol), [^{3}H]estradiol (85–99 Ci/mmol) were from Amersham Radiochemical Center (Bucks, U.K.). Calibration kit for the electrophoresis of low mol. wt proteins was from Pharmacia (Uppsala, Sweden). Acrylamide, BIS, TEMED, Ammonium Persulfate, gelatine and Norit A were from Serva (Heidelberg, Germany). All other reagents were of analytical grade.

Buffers

The following buffers were used: 50 mM Tris–HCl, 1 mM dithiothreitol pH 7.4, without (TD buffer) or with 0.2 mM EGTA (TGD buffer).

Calf uterus estrogen receptor purification

Calf uterus ER was purified according to a previously described procedure [12].

Calf uterus kinase purification

This enzyme was purified according to the procedure recently reported which utilizes as a final step a Calmodulin–Sepharose chromatography [10]. 30–50 g uteri frozen and stored in liquid nitrogen for no longer than 1 week were used. Only cytosols containing at least 500–600 fmol of [^{3}H]estradiol binding sites/mg protein were utilized as a source of the enzyme. The enzymic activity is expressed in units (U) as previously published [10].

In vitro expression of HEO and HEGO receptors

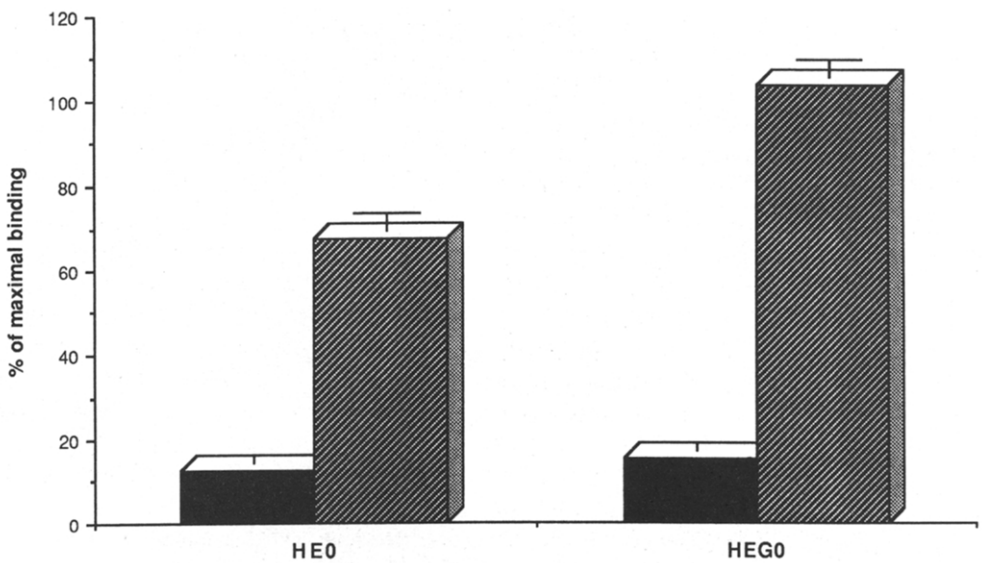
The HEO receptor cDNA was subcloned in Bluescribe M13^{+} and HEGO receptor cDNA was subcloned in a pBluescript SK^{+} vector at EcoRI site downstream to the promoter T7. HEO DNA and HEGO DNA were linearized as previously described [10] using BamHl and Sall, respectively. The transcription was performed according to a previously reported procedure [9] using T7 polymerase. *In vitro* translation was made using rabbit reticulocyte lysate (Amersham N 90 or Promega) and 5 μg mRNA for 50 μl incubation mixture. Incubation was at 30°C for 90 min and the amount of synthesized protein was determined by incorporation of [^{35}S]methionine in an aliquot of the reaction. The amount of the synthesized receptor was estimated from methionine pool size, the amount of [^{35}S]methionine specifically incorporated after subtraction of background and the number of methionine residues of the receptor molecule [9].

Assay of specific [^{3}H]estradiol binding sites of synthetic receptors under basal conditions

50 μl lysate containing *in vitro* synthetized HEO or HEGO was diluted to 230 μl with TGD-buffer and then divided into two 100 μl fractions and incubated at 4°C overnight with 10 nM [^{3}H]estradiol in the absence and in presence of a 100-fold excess of radioinert hormone. Thereafter 200 μl of dextran-coated charcoal (1% activated charcoal, 0.01% dextran T 100, 0.5% gelatin in 50 mM Tris, pH 7.4) were added. Samples were centrifuged after 5 min incubation at 4°C and 100 μl of supernatants were counted in duplicate. Specific binding was calculated by subtracting non specific from total binding.

Activation of synthetic receptors and assay of specific [^{3}H]estradiol binding sites after kinase activation

Activation of hormone binding of the *in vitro* synthesized receptor by kinase was performed by a modification of a previously reported procedure [5]. Identical mixtures were prepared in TGD-buffer with the following composition: 50 μl lysate containing *in vitro* synthesized HEO or HEGO estradiol receptor, 90 μl of TGD buffer containing where indicated 200–250 fmol of calf uterus, dextran coated charcoal treated [^{3}H]estradiol (10 Ci/mmol)–receptor complex, 90 μl of purified kinase preparation corresponding to 2–4 arbitrary units of enzymic activity [10], 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 μg/ml calmodulin in a final volume of 240 μl. 100 μl aliquots from each mixture were labeled with 10 nM [^{3}H]estradiol in the absence (total binding) and in presence of a 100-fold excess of radioinert hormone (non-specific binding). After 0.5–2 h at 0°C each sample was incubated with 10 μl of 50 mM ATP at 15°C for 10 min. Samples were then cooled, incubated overnight at 4°C, added with 200 μl dextran-coated


120
100
80
60
40
20
0
% of maximal binding
HE0
HEG0

pears if the kinase is omitted from the incubation mixture used to activate synthetic receptors (not shown). Therefore the difference is due to the kinase activity.

In one experiment we have incubated the two synthetic receptors separately with the kinase under conditions identical to those used to activate the synthetic receptors (see Methods) except for replacing 5 mM ATP with 50 μM [^{32}P]ATP and followed ^{32}P-phosphorylation of receptors. The results of these experiments are reported in Fig. 2. Both, HEO (lane 2) and

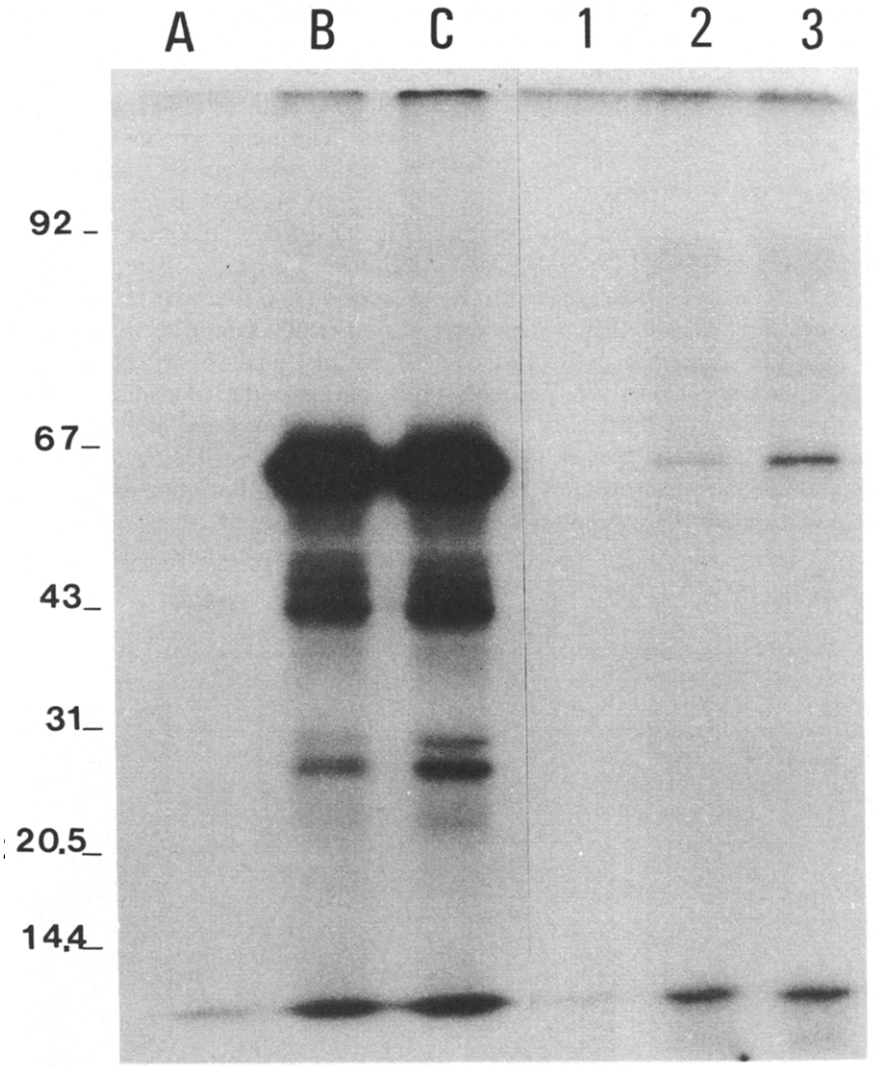


Fig. 2. SDS–PAGE analysis of the kinase phosphorylated HEO and HEGO synthetic receptors. Rabbit reticulocyte lysate 50 μl aliquots were incubated without or with 5 μg of either HEO or HEGO mRNA at 30°C for 90 min. At the end of this incubation the aliquots were added in the cold with about 2 U of purified kinase and 120 fmol of charcoal treated purified [^{3}H]estradiol(10 Ci/mmol)-calf uterus receptor complex in 200 μl of TGD-buffer and incubated at 15°C for 10 min in the presence of 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 μg/ml calmodulin and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). At the end of incubation the samples were cooled, added to 10 μl ascites containing JS34/32 antireceptor antibody and 15 μl 10% Pansorbin suspension and incubated at 4°C for 2 h in the presence of 1 mM phenyl–methyl–sulphonyl–fluoride by gentle shaking. Samples were then layered on the top of 1 ml of 1 M Sucrose containing 1% deoxycholate and centrifuged at 13,000 rpm in an Eppendorf centrifuge for 5 min. The Pansorbin pellet was collected, suspended in 200 μl of 50 mM Tris–HCl, added with 0.5% deoxycholate and centrifugation through sucrose was repeated once again. The final pellet was suspended in a small volume of Laemmli SDS–PAGE sample buffer, boiled, centrifuged and clear supernatant subjected to SDS–PAGE as reported in methods. Dried slabs were submitted to autoradiography. Lanes 1–3 show the phosphorylation pattern of the proteins translated by rabbit reticulocyte lysate without (lane 1), and with HEO (lane 2) or HEGO mRNA (lane 3). Lanes A, B, C show the fluorography of electrophoresis of 5 μl aliquots of [^{35}S]methionine labeled proteins synthesized in the lysate in the absence (lane A) or in presence of either HEO mRNA (lane B) or HEGO (lane C), respectively. Parallel samples of HEO and HEGO incubated in the same conditions but using 5 mM radioinert nucleotide instead of [γ-^{32}P]ATP increased their hormone binding activity from 9 to 58% and from 8 to 99% of maximal binding activity, respectively.

HEGO (lane 3) appear to be phosphorylated by the kinase. The specificity of this phosphorylation is shown by the absence of a 67 kDa ^{32}P-phosphorylated protein in the immunoprecipitate of a lysate which did not synthesize ER (lane 1). Therefore, HEGO during hormone binding activation is phosphorylated. In Fig. 2 HEGO appears to be more phosphorylated than HEO. This can be due to the fact that HEGO behaves as a better kinase substrate than HEO as shown by data on activation of binding sites reported in the legend to Fig. 2 as well as to Fig. 1. This difference at least in part could be also attributed to a major lability of HEO [11], which might be responsible for a partial loss of phosphorylated receptor during the time-consuming procedure required by immunoprecipitation and washing of immunoprecipitated receptor.

HEGO activates the tyrosine kinase

The uterus tyrosine kinase responsible for the activation of ER binding sites in cell-free systems shows the interesting property to be significantly stimulated by estradiol in complex with the uterus hormone binding receptor [6]. This stimulation is due to the fact that hormone–receptor complex strongly increases the affinity of the enzyme for the substrate which is the dephosphorylated receptor unable to bind hormone [6]. Experiments on the kinase activation of the mutant HEO showed that this activation was extensive only in the presence of calf uterus ER preloaded with hormone [10]. In contrast, present findings show that the kinase does not require addition of calf uterus estrogen–receptor complex to activate HEGO hormone binding. In fact HEGO like calf uterus receptor is able to significantly stimulate the kinase in the presence of hormone.

As can be observed in Fig. 3, the basal hormone binding activity of HEO and HEGO in the lysate is almost the same: 16 and 15% of their maximal theoretical hormone binding capacity (1 mol of hormone bound/1 mol of synthesized receptor), respectively (Bars A). When the kinase is added to the synthetic receptors, preincubated at 0°C with [^{3}H]estradiol for 0.5 h, together with ATP and other compounds used to activate the synthetic receptors, but in the absence of estradiol–calf uterus receptor complex, hormone binding activity of HEO receptor (Bar B) is not different from that

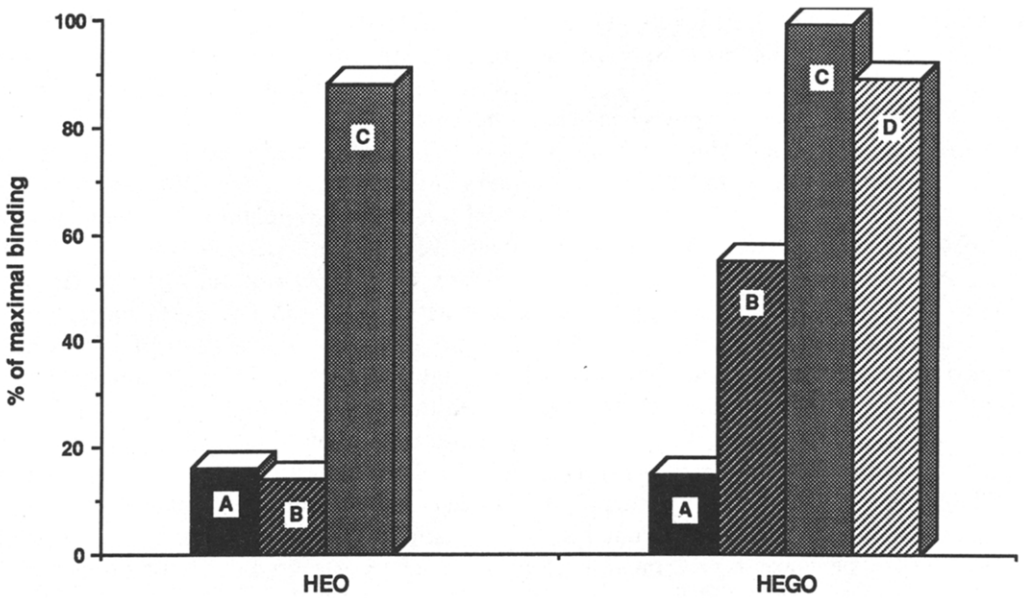


Fig. 3. Stimulation of the uterus kinase by estradiol–HEGO complex. Estradiol receptor was synthesized by incubating 3 aliquots of 50 μl rabbit retyculocyte lysate at 30°C for 90 min with 5 μg of either HEO or HEGO receptor mRNA. One of these aliquots was used to assay specific [^{3}H]estradiol binding sites under basal conditions as reported in Methods. Each of the remaining aliquots was incubated with 90 μl of purified kinase (about 4 U) then used to follow kinase activation of synthetic receptors and assay specific [^{3}H]estradiol binding sites after kinase activation under conditions identical to those reported in methods except for the absence of [^{3}H]estradiol–calf uterus receptor complex (E_2R) in columns B and D. The columns show the percent of maximal binding of HEO and HEGO under basal conditions (A) and after kinase activation either without E_2R using synthetic receptors preincubated with 10 nM [^{3}H]estradiol for 0.5 h (B) or 2 h (D) or with E_2R for the 0.5 h (C). The amounts of receptor synthesized in 10 μl of lysate are the following: HEO: 112 fmol; HEGO 118 fmol.

assayed under basal conditions, whereas HEGO receptor hormone binding activity increases up to 55% of the maximal binding activity (Bar B). HEGO hormone binding after kinase activation reaches 90% of the maximal binding (Bar D) when the preincubation time at 0°C of this receptor with [^{3}H]estradiol is shifted from 0.5 to 2 h. This shift allows a more complete equilibration of HEGO with estradiol and therefore a more pronounced stimulation of the kinase. It should be pointed out that this hormone binding increase of HEGO is not observed in the absence of estradiol (not shown). This confirms that the liganded receptor only is able to stimulate the kinase [6]. Also in the experiment presented in Fig. 3, as peviously shown in Fig. 1, activation of binding sites of both receptors HEO and HEGO by the kinase is observed in the presence of estradiol–calf uterus receptor complex (Bars C).

In conclusion HEGO, like calf uterus receptor and in contrast with HEO, stimulates the kinase when it is in the presence of estradiol.

DISCUSSION

Phosphorylation is the reversible post-translational modification which most frequently modulates protein function [13]. It often occurs on serine and threonine whereas it is very rare on tyrosine [14].

Phosphorylation of steroid receptors has been observed in cells, tissues and animals under a variety of conditions [15]. Nevertheless, the functions of these phosphorylations are still unclear. ER was the first nuclear protein reported to be phosphorylated on tyrosine [2]. Other nuclear proteins have thereafter been found to share this feature with ER. In murine transformed fibroblasts nuclear proteins phosphorylated on tyrosine which bind preferentially to murine DNA have been found [16]. The LDH-5 isoenzyme is present in nuclei [17], is a DNA binding protein [18] and in nuclei of pheochromocitoma cells is phosphorylated on tyrosine [19]. Also the nuclear protein pp64 is phosphorylated on tyrosine in rat kidney cells [20].

Hormone binding of uterus ER is regulated by tyrosine phosphorylation-dephosphorylation in cell-free systems [1–6]. The finding that ER in uterus is phosphorylated on tyrosine and interacts with antiphosphotyrosine antibody [2] corroborates the physiological role of the ER phosphorylation on tyrosine. The cloned human estrogen receptor HEO binds estradiol at 4°C with high affinity, but only a small percentage of the receptor molecules synthesized *in vitro* binds hormone [9, 19]. Incubation of HEO with the uterus estradiol receptor tyrosine kinase activates most of the non-hormone binding receptor [10].

However, the cloned human estrogen receptor HEO contains a mutation (valine substitutes for glycine at position 400) which alters its hormone binding properties at 25°C. In addition, in the absence of estradiol this receptor is labile at this temperature [11]. These findings raised the possibility that the low efficiency of binding at 4°C of HEO is a consequence of the mutation at position 400 and that stimulation of binding activity by the kinase is due to a "repair" effect of phosphorylation on the mutated receptor. This possibility, which did not appear very likely since it was already known that the kinase activates hormone binding of a wild type calf uterus receptor [1, 6], is excluded by the present results. HEGO, also binds estradiol with low efficiency suggesting that a post-translational modification which is either absent or present at very low level in the ER of the lysate is required for hormone binding. This is proved by the finding that incubation of HEGO with the uterus tyrosine kinase produces both phosphorylation and activation of binding sites of this receptor which is an excellent substrate of the kinase.

HEGO is able to stimulate the kinase and this stimulation occurs only in the presence of estradiol. A similar stimulation has been observed with the calf uterus receptor [6].

Why does HEO not activate the kinase, in contrast to HEGO? The exact mechanism of hormone-receptor stimulation of the kinase is still unknown. Nevertheless it can be hypothesized that the mutation at position 400 induces a conformational change of the receptor. Because of this change ER might not exert an efficient stimulation of the kinase.

Hormone stimulated tyrosine kinases are intrinsic to peptide growth factor receptors [21]. In the case of ER no intrinsic kinase is detectable [15]. Nevertheless, estradiol binding to ER stimulates a tyrosine kinase. We believe that the estradiol–receptor complex binds to the tyrosine kinase producing the observed drastic increase of affinity of the enzyme for the non-hormone binding receptor [6]. This model should be verified in estradiol responsive cells and tissues since the *in vitro* observed stimu-

lation of a tyrosine kinase by estradiol [6, 10 and present paper] raises the possibility that, similar to peptide growth factors and other mitogenic signals, estradiol stimulates cell growth through activation of tyrosine phosphorylation.

Functional association of membrane receptors with ligand-activated tyrosine kinases have been reported [22–24], but this is the first association described for an intracellular receptor.

Acknowledgements—We are indebted to Dr Bruno Moncharmont for JS 34/32 antibody, to Mr Domenico Piccolo for technical assistance and to Mr Gian Michele La Placa for editorial work.

This research was supported by grants from Associazione Italiana per la Ricerca sul Cancro, from Consiglio Nazionale delle Ricerche Italiano, Progetto Speciale Oncologia, contratto no. 88.505.44 and from Ministero Pubblica Istruzione.

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