Properties of a Purified Estradiol-Dependent Calf Uterus Tyrosine Kinase†

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ABSTRACT: A uterus tyrosine kinase has been purified to a single 67-kDa protein when analyzed by SDS–PAGE. Under nondenaturing conditions the molecular weight of the enzyme ranges from 114 to 136 kDa, depending on the procedure employed. The kinase binds calmodulin in a Ca^{2+}-dependent manner and the ATP analog [(fluorosulfonyl)benzoyl]adenosine. The purified enzyme phosphorylates the phosphatase-treated uterus estradiol receptor on tyrosine and activates its hormone binding. The kinase phosphorylates actin, calmodulin, and histone H2B. Whatever the substrate, the enzymic activity is dependent on purified estradiol–receptor complex and is activated by Ca^{2+}-calmodulin. The kinase activates and phosphorylates the human estradiol receptor (HEO) within the hormone binding domain (HBD) [Migliaccio, et al. (1989) *Mol Endocrinol. 3*, 1061–1069] as well as four of the five mutants of the HEO obtained by substituting each of the five tyrosine residues present in the HBD of the receptor with phenylalanine by site-directed mutagenesis. The mutant substituted at tyrosine 537 is the only one that is neither phosphorylated nor activated by the kinase. This proves a causal relationship between the phosphorylation of estradiol receptor on tyrosine 537 and its hormone binding activity. A synthetic peptide corresponding to 11 out of 13 amino acids surrounding tyrosine at position 537 of the human estrogen receptor can be phosphorylated by the kinase. This and other findings indicate that this kinase, unlike other tyrosine kinases, phosphorylates tyrosyl residues with acidic amino acids close to the carboxyl side.

All steroid receptors are phosphorylated under physiological conditions. The role of this phosphorylation is actively investigated. Transformation, hormone binding, DNA binding, and transactivation of steroid receptors have been proposed to be functional targets of receptor phosphorylation (Auricchio, 1989; Carson-Jurica et al., 1990; Moudgil, 1990; Jensen, 1991; Orti et al., 1992).

Using calf uterus cytosol, we identified an enzyme which after partial purification is able to confer hormone binding with physiological affinity to the estradiol receptor either synthesized in vitro (Migliaccio et al., 1989, 1991) or purified from calf uterus and dephosphorylated (Migliaccio et al., 1984). During activation of the binding, tyrosine phosphorylation occurs. In addition, in uteri incubated in the presence of orthovanadate, the cytosol receptor has been found to be phosphorylated on tyrosine (Migliaccio et al., 1986). Other members of the nuclear receptor superfamily have been found to be phosphorylated on tyrosine: the glucocorticoid receptor (Rao & Fox, 1987) and the β_1 thyroid hormone receptor (Lin et al., 1992).

We now report that the uterus enzyme with its unique ability to confer hormone binding to the estradiol receptor has been extensively purified. Under nondenaturating conditions the kinase seems to be a dimer of a 67-kDa protein. Other proteins in addition to the nonhormone binding receptor have also been found to be substrates of the tyrosine kinase. Whatever the substrate, the activity of the kinase is dependent on the estradiol–receptor complex. Ca^{2+}-calmodulin stimulates the phosphorylating activity. We have previously observed that tyrosine phosphorylation by the kinase occurs exclusively within or near the HBD of a synthetic estradiol receptor (Migliaccio et al., 1989). The use of point-mutated synthetic estradiol receptors shows that tyrosine at position 537 is necessary for both hormone binding activation and phosphorylation of the receptor by the kinase. A synthetic peptide corresponding to the sequence of the receptor around tyrosine at position 537 can be phosphorylated by the kinase.

MATERIALS AND METHODS

Reagents. [^{3}H]Estradiol (99 Ci/mmol), [γ-^{32}P]ATP (3000 or 10 Ci/mmol), [^{35}S]methionine (800 Ci/mmol), rabbit reticulocyte lysate, rainbow markers for low molecular weight protein electrophoresis, and Amplify were from Amersham, Buckinghamshire, U.K.; [^{125}I]calmodulin (50–150 μCi/μg) and [^{14}C]FSO_4-Bz-Ado (40–60 mCi/mmol) were from Du Pont de Nemours, Bad Homburg, GmbH, Germany. Calmodulin from bovine brain, actin from chicken muscle, soybean trypsin inhibitor, poly(Glu-Tyr) 4:1, angiotensin II, and ATP–agarose were from Sigma, St. Louis, MO. Sepharose 4B, calmodulin–Sepharose, and the calibration kit for low molecular weight protein electrophoresis were from Pharmacia, Uppsala, Sweden. Acrylamide, Bis, TEMED,[1] ammonium persulfate, SDS, Coomassie Brilliant Blue R 250, and the kit for silver staining of proteins were from Bio Rad, Richmond, CA. DE 52 cellulose, CM cellulose, and phosphocellulose filters P81 were from Whatman, Springfield Mill, Madison, Kent, U.K. Cellulose nitrate (0.45 μm) was from Schleicher & Schuell, Dassel, Germany. Monoclonal anti-phosphotyrosine antibodies IgG2bk (clone 4 G10) were from UBI, Lake

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Placid, NY. Anti-mouse IgG-AP conjugate, Tween 20, a kit for Western blot detection of proteins with the ProtoBlot AP system, and rabbit reticulocyte lysate were from Promega, Madison, WI. All other reagents were of analytical grade. Peptide K13E was synthesized and purified to 97% homogeneity by Peninsula Laboratories Ltd., Mersey Side, U.K.

Kinase Purification. (*a*) *Cytosol Preparation.* The enzyme was purified from uteri of young calves weighing 10–80 g each. Uteri were frozen in liquid nitrogen and stored for no longer than 1 week. Uteri with at least 700 fmol of [^{3}H]-estradiol specific binding sites/mg of protein were used. Cytosols with low receptor levels contain low kinase levels. Tissue (50 g) was homogenized in 4 vol of cold TGD buffer with an Ultraturrax homogenizer (Janke & Kunkel, Staufen, Germany) using four bursts at 70 V, lasting 10–20 s each, at 1-min intervals. The homogenate was filtered through cheesecloth and then centrifuged at 105000*g* for 45 min at 2 °C in a L8-55 Beckman centrifuge equipped with a Ti 50.2 rotor.

(*b*) *Ammonium Sulfate Fractionation.* $(NH_4)_2SO_4$ was added at 2 °C to the cytosol up to 25% of saturation. After 45 min of standing and 20 min of centrifugation at 30000*g* at 2 °C most of the estradiol receptor was found in the pellet. Additional $(NH_4)_2SO_4$ was added to the supernatant up to 50% saturation, and after 2 h the suspension was centrifuged at 30000*g* for 20 min at 2 °C. The pellet was solubilized in TGD buffer, clarified by centrifugation, and then diluted with TGD buffer to reach a conductivity value of 4 $m\Omega^{-1}$.

(*c*) *Cycling through Heparin–Sepharose Column.* The sample was applied on a 5-mL heparin–Sepharose column preequilibrated with TGD buffer and cycled overnight at 2 °C at a flow rate of 30 mL/h. This step separates the kinase from the residual estrogen receptor present in the preparation.

(*d*) *DEAE-Cellulose Chromatography.* The flow-through from the heparin–Sepharose column was diluted with TGD buffer to reach a conductivity value of 2–2.5 $m\Omega^{-1}$ and poured on a 30-mL DEAE-cellulose column preequilibrated with TGD buffer. The column was washed with the same buffer until the optical density at 280 nm of the washing buffer was equal to that of the equilibration buffer. The kinase was then eluted from the column using a 0–0.4 M KCl linear gradient (four times the volume of the column). Fractions (1.5 mL) were collected and monitored for enzyme activity. Fractions containing the peak of enzymic activity were pooled.

(*e*) *Calmodulin–Sepharose Chromatography.* The pooled fractions were diluted with TGD buffer to reach a concentration of 0.1 M KCl. $CaCl_2$ was added to a final concentration of 1 mM. The sample was applied to 2.5-mL calmodulin–Sepharose column preequilibrated with TGD buffer containing 0.1 M KCl and 1 mM $CaCl_2$, at a flow rate of 15 mL/h. The column was initially washed with 30 vol of the equilibration buffer and then with 2 vol of TD buffer containing 0.1 M KCl and 2 mM EGTA, pH 7.4. The enzyme was eluted from the column with 4 mL of TD buffer containing 0.1 M KCl and 5 mM EGTA, pH 7.4.

(*f*) *Poly(Glu-Tyr) 4:1-Sepharose Chromatography.* The eluate from calmodulin–Sepharose was diluted with TGDM buffer to 0.05 M KCl and then incubated batchwise at 2 °C for 2 h with 1.5 mL of poly(Glu-Tyr) 4:1-Sepharose preequilibrated with TGDM buffer. The slurry was poured on a column, washed with 20 vol of the preequilibration buffer, and finally eluted with 2 vol of TGDM buffer containing 0.4 M KCl, pH 7.4. The eluate was collected and immediately diluted with TGDM buffer to 0.05 M KCl.

(*g*) *ATP–Agarose Chromatography.* The eluate from poly-(Glu-Tyr) 4:1–Sepharose was incubated batchwise at 2 °C for 90 min with 1.25 mL of ATP–agarose equilibrated with TGDM buffer. The slurry was then poured on a column, washed with 20 vol of the equilibration buffer, and finally eluted with 2 vol of TGDM buffer containing 0.8 mM $CaCl_2$ and 5 mM ATP, pH 7.4. Glycerol was added to a final concentration of 40%, and the enzymic preparation was stored at −20 °C for no longer than 3 days. ATP was removed from the kinase added with 0.3 M KCl by passage through a 1.25-mL DEAE-cellulose column preequilibrated with TGD buffer containing 0.3 M KCl. The sample was dialyzed against TGD buffer (ATP-free purified kinase) and used. Protein concentration was determined using a Bio Rad protein assay kit.

Crude Phosphatase-Inactivated Uterus Estradiol Receptor. Estradiol receptor was precipitated from cytosol with 25% ammonium sulfate, equilibrated with 20 nM [^{3}H]estradiol in the absence and presence of excess cold estradiol and its hormone binding activity partially inactivated by a uterus nuclear phosphatase (Auricchio et al., 1987a).

Routine Enzymic Activity Assay. This was based on the ability of the uterus kinase to activate the phosphatase-inactivated estradiol receptor. Aliquots of the kinase preparation (200–250 μL) were incubated with an equal volume of TGD buffer containing crude phosphatase-inactivated estradiol receptor. Reaction was performed at 15 °C for 10 min in the presence of 10 mM Na_2MoO_4, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 10 μg/mL calmodulin, with or without 5 mM ATP. After incubation, samples were cooled at 2 °C for 3 h and then assayed for [^{3}H]estradiol receptor specific binding sites by DCC treatment (Auricchio et al., 1987a). Kinase-activated binding sites of estradiol receptor were computed from the difference between the [^{3}H]estradiol specific binding sites of the samples incubated with and without ATP.

Purification of Estradiol Receptor and Nuclear Phosphatase. [^{3}H]Estradiol (10 Ci/mmol)–receptor complex and nuclear phosphatase were purified according to previously reported procedures (Castoria et al., 1988; Auricchio et al., 1981b).

Reversible Denaturation of the Kinase. The purified kinase preparation (2.5 mL) was dialyzed in the presence of electrophoretically purified soybean trypsin inhibitor (50 μg) at 2 °C against TD buffer using a SpectraPor 7 dialysis membrane (Spectrum, Los Angeles, CA). A control sample containing 50 μg of purified soybean trypsin inhibitor in 2.5 mL of TD buffer but no kinase was submitted to the same procedure in parallel. The samples were lyophilized in 0.1% SDS (w/v), solubilized in 50 μL of Laemmli sample buffer, and analyzed in duplicate by 11% SDS–PAGE. One lane was submitted to silver nitrate staining; the other lane was renatured according to a previously described procedure (Hager & Burgess, 1980) with minor modifications. Soybean trypsin inhibitor (0.1 mg/mL) was used as a carrier instead of bovine serum albumin when proteins were eluted from

[1] Abbreviations: ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; AMP-PNP, 5′-adenylylimidodiphosphate; FSO_4-Bz-Ado, [(fluorosulfonyl)benzoyl]adenosine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; TD buffer, 10 or 50 mM Tris–HCl/1 mM DTT, pH 7.4; TGD buffer, TD buffer containing 0.2 mM EDTA; TGDM buffer, TGD buffer containing 5 mM $MgCl_2$; TBST buffer, 10 mM Tris–HCl/150 mM NaCl/0.05% Tween 20 (v/v) pH 8; AP buffer, 0.1 M Tris–HCl/0.1 M NaCl/5 mM $MgCl_2$, pH 9.5; DCC, dextran-coated charcoal; HEO, human synthetic estrogen receptor; HE-F, point mutated HEO; HBD, hormone binding domain.

acrylamide slices. Proteins were precipitated with acetone and then dissolved in 50 μL of cold dilution buffer (0.05 M Tris–HCl, 0.15 M NaCl, 20% glycerol (v/v), 1 mM DTT, and 0.1 mM EDTA, pH 7.9) containing 6 M guanidine hydrochloride. Dilution buffer 2.5 mL containing 10 mM Na_2MoO_4, 5 mM $MgCl_2$, 10 μg/mL calmodulin, and 0.8 mM $CaCl_2$ was added, and the samples were gently mixed and left at 4 °C for 18 h. A 400-μL aliquot from each sample was incubated with 200 μL of TGD buffer containing crude phosphatase-inactivated estradiol receptor under conditions described in the routine enzymic activity assay section but omitting the samples incubated without ATP. Kinase-activated binding sites of estradiol receptor were determined as the difference between the specific binding sites of the samples obtained from the slices of the gel lane loaded with trypsin inhibitor and the kinase and the specific binding sites of parallel samples obtained from the slices of the gel lane loaded only with trypsin inhibitor (control).

Protein Tyrosine Kinase Assay. (*a*) *^{32}P Phosphorylation of Purified Estradiol Receptor.* One milliliter of TD buffer containing ~1200 fmol of [^{3}H]estradiol–receptor was incubated at 25 °C for 15 min with 0.5 mL of purified uterus nuclear phosphatase. A 900-μL aliquot of phosphatase-inactivated estradiol receptor was incubated with the purified kinase (300 μL) as previously described (see Routine Enzymic Activity Assay section). The mixture was then divided into two fractions, and only one was supplemented with 1.8 mM EGTA. Each fraction was divided in two aliquots. One (300 μL) was incubated with 0.15 mM radioinert ATP to assay the kinase-induced hormone binding reactivation. The other (200 μL) was incubated with 0.15 mM [γ-^{32}P]ATP and then immunoprecipitated overnight at 4 °C with 20 μL of rat ascites containing JS 34/32 antireceptor antibodies (Moncharmont et al., 1982) and 15 μL of 10% Pansorbin suspension. The samples were then poured over a 1% deoxycholate cushion with 1 M sucrose in 50 mM Tris–HCl, pH 7.4. The pellets were solubilized with Laemmli sample buffer (50 μL) and analyzed by 11% SDS–PAGE.

(*b*) *^{32}P phosphorylation of histone H_2B and calmodulin* was followed by mixing 100 μL of ATP-free purified kinase with 100 μL of TGD buffer containing ~100 fmol of purified [^{3}H]estradiol–receptor complex. The incubation mixture contained, in a final volume of 250 μL, 100 μM orthovanadate (freshly solubilized) and 5 mM $MgCl_2$. When indicated, 210 μM $CaCl_2$ and 10 μg/mL calmodulin were added to the mixture. The histone H_2B concentration was 10 μM. When calmodulin was used as a substrate, its concentration was shifted to 50 μg/mL. For each substrate, whether lacking kinase, [^{3}H]estradiol–receptor complex, or substrate, control samples were incubated in parallel. The reaction was started with 50 μM [γ-^{32}P]ATP, continued at 20 °C for 20 min, and then stopped by adding cold TCA (10% v/v). Proteins were precipitated at 2 °C for 12 h, pelleted at 5000 rpm for 30 min, and then washed three times with 1 mL of ether–ethanol (1:1 v/v). The pellets were solubilized with Laemmli sample buffer (50 μL) and analyzed by a 18% SDS–PAGE. The gels containing ^{32}P-phosphorylated proteins were fixed, dried, and autoradiographed at −80 °C using Kodak X-OMAT AR films in Dupont Cronex Lightning cassettes with intensifying screens.

(*c*) *Actin phosphorylation* was performed by mixing 250 μL of ATP-free purified kinase with 250 μL of TGD buffer containing 100 fmol of purified [^{3}H]estradiol–receptor. The mixture contained, in a final volume of 500 μL, 100 μM sodium orthovanadate (freshly solubilized), 5 mM $MgCl_2$, 10 μg/mL calmodulin, and 0.15 mM ATP. The reaction was started by adding 5 μg/mL chicken muscle actin and continued at 15 °C for 20 min. When indicated, 210 μM $CaCl_2$ was added to the incubation mixture. As for calmodulin and histone H_2B, appropriate control samples were incubated in parallel. The reaction was stopped by adding cold TCA (10% v/v). Precipitated proteins were washed with ether–ethanol (1:1 v/v) and solubilized with Laemmli sample buffer (50 μL). Aliquots (20 μL) of each sample were simultaneously analyzed by 11% SDS–PAGE using two separate gels and then transferred to nitrocellulose as previously described (Kyshe-Andersen, 1984). The membranes were incubated for 2 h at room temperature in blocking solution [10 mM Tris–HCl containing 150 mM NaCl, 3% bovine serum albumin (w/v), and 0.02% sodium azide (v/v), pH 8]. One of two membranes was immunoblotted with 20 μg of monoclonal anti-phosphotyrosine antibodies in 20 mL of blocking solution containing 20 mM phosphoserine and 20 mM phosphothreonine. The other membrane was hybridized under the same conditions except in the presence of 20 mM phenyl phosphate. Immunoblotting was carried out at room temperature for 12 h under gentle shaking. After three washings with TBST buffer, the membranes were incubated with goat antimouse IgG conjugated to alkaline phosphatase and the antibody-coupled phosphatase activity was revealed by BCIP and NBT in AP buffer according to the manufacturer's instructions.

(*d*) *^{32}P phosphorylation of peptides* K13E, E11G1, and angiotensin II was carried out under the conditions described for histone H_2B. The substrate concentration was 2 mM. The reaction was stopped by adding cold TCA (2.5% v/v), and samples were centrifuged at 5000 rpm for 20 min. Aliquots (50 μL) of supernatant from the samples containing angiotensin II and peptide E11G1 were spotted in duplicates onto phosphocellulose P81 filters. Filters were washed and dried as previously described (Glass et al., 1978; Swarup et al., 1983) and then counted for radioactivity. Phosphorylation of angiotensin II and E11G1 peptides was also assayed by high-voltage electrophoresis (Wong & Goldberg, 1983). In the case of peptide K13E, after TCA addition and centrifugation, the pellets were collected and washed four times with cold TCA (2.5% v/v) containing 20 mM sodium pyrophosphate. They were washed four additional times with cold ether–ethanol (1:1 v/v). Pellets were suspended in MilliQ H_2O, and 50-μL aliquots were counted in duplicate for radioactivity. For each enzyme sample, appropriate reaction mixtures containing no peptide were run as controls. Kinase activity was determined from the difference between radioactivity of the samples incubated with and without peptide.

Site-Directed Mutagenesis on HEO-pSG-4 Plasmid and in Vitro Expression of HEO and HE-F Mutants. Single-stranded DNA templates were prepared using the expression vector pSG4 (Kumar et al., 1986) containing the 1.8-kb insert that has the ER cDNA complete open reading frame (HEO-ORF) (Green et al., 1986). Five oligonucleotide primers for the site-directed mutagenesis were designed so that the tyrosine codons corresponding to positions 328, 331, 459, 526, and 537 of the ER molecule were changed into phenylalanine codons (HE-F mutants), and a novel restriction site was created for each mutant: a *Ssp*I site in HE328, a *Stu*I site in HE331, a *Xho*I site in HE459 and HE537, and a *Eag*I site in HE526. The mutants were screened using the 5′-end-labeled mutagenesis oligonucleotide as a probe, and all the constructions were verified by selective cleavage using the above-mentioned restriction enzymes and checked by dideoxy sequencing. The tyrosine–phenylalanine-mutated receptor cDNA were cloned

Table I: Purification of the Uterus Kinase

steps[a]	vol (mL)	total protein (mg)	total activ (arb units)[b]	purif factor	yield (%)
heparin–Sepharose break through	50	189.9	109	1	100
DEAE-cellulose	10	2.87	34	21	31
calmodulin–Sepharose	4	<0.005[c]	28	>5660[c]	25
poly(Glu-Tyr)–Sepharose	3		27		24
ATP–agarose	2.5		26		23

[a] The initial two steps, cytosol preparation and ammonium sulfate fractionation, are not reported in the table because the activity was not assayed at these stages. [b] One arbitrary unit is the activity activating 1×10^{-14} specific binding sites per minute (Auricchio et al., 1981a). [c] Protein concentration is below the lowest limit of the assay (1 μg/0.8 mL). Maximal protein concentration and minimal purification factor of the uterus kinase eluted from calmodulin–Sepharose are calculated on this basis. Also, in the last two purification steps protein concentration was not assayable.

at the *Eco*RI site in pSG4 vector. In all cases, the DNA was linearized as previously described using *Bam*HI and transcription performed according to the same report using T7 polymerase (Kumar et al., 1986). In vitro translation was performed as above described (Migliaccio et al., 1989) and the amount of synthesized protein determined from the incorporation of [^{35}S]methionine in an aliquot of the reaction. The amount of the synthesized receptor was estimated according to a previous report (Kumar et al., 1986).

Hormone Binding Activation and ^{32}P Phosphorylation by the Uterus Kinase of HEO and HE-F Mutants. Lysate (100 μL) containing translated receptor was incubated with 180 μL of the kinase eluted from calmodulin–Sepharose and 180 μL of purified charcoal-treated [^{3}H]estradiol (10 Ci/mmol)–receptor complex (containing ~400 fmol) in the presence of 5 mM $MgCl_2$, 0.8 mM $CaCl_2$ and 10 μg/mL calmodulin. Aliquots (100 μL) from each mixture were labeled in duplicate with 10 nM [^{3}H]estradiol (99 Ci/mmol) in the absence and in the presence of a 100-fold excess of radioinert hormone. The samples were left ~30 min at 0 °C then incubated at 15 °C for 10 min in the absence or in the presence of 5 mM ATP. After incubation, samples were cooled at 2 °C for 12 h and then assayed for [^{3}H]estradiol receptor binding sites by DCC treatment. Kinase-activated binding sites of HEO and HE-F mutants were computed as reported in the routine enzymic activity assay section. ^{32}P phosphorylation of HEO and HE-F mutants was carried out by incubating 50-μL aliquots of reticulocyte lysate containing translated receptor with 90 μL of the kinase eluted from calmodulin–Sepharose and 150 fmol of charcoal-treated purified [^{3}H]estradiol (10 Ci/mmol)–calf uterus receptor complex in 200 μL of TGD buffer. The phosphorylation mixture contained 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 μg/mL calmodulin, and 10 nM [^{3}H]estradiol. The reaction was started by adding 50 μM [γ-^{32}P]ATP (10 Ci/mmol) and continued at 15 °C for 10 min. The control sample containing 50 μL of receptorless rabbit reticulocyte lysate was incubated in parallel. The samples were then immunoprecipitated at 4 °C for 2 h with 10 μL of rat ascites containing JS34/32 antireceptor antibody and 15 μL of 10% Pansorbin suspension containing 1 mM PMSF (freshly solubilized). Samples were then layered on top of a 1-mL cushion of 1 M sucrose and 1% deoxycholate in 50 mM Tris–HCl, pH 7.4, and then centrifuged at 13 000 rpm for 5 min. The pellets were suspended in 200 μL of 50 mM Tris–HCl containing 0.5% deoxycholate. Centrifugation through the sucrose gradient was repeated once. Finally, the pellets were solubilized with Laemmli sample buffer (50 μL) and analyzed by 11% SDS–PAGE. ^{32}P proteins were detected by autoradiography.

[^{32}P]Phosphoamino Acid Analysis. The ^{32}P-phosphorylated estradiol receptor or calmodulin was extracted from gel slices, hydrolyzed in 6 N HCl at 110 °C for 90 min, and then submitted to phosphoamino acid analysis as previously reported (Auricchio et al., 1987a) except that the electrophoresis was run in only one direction (pH 3.5).

Polyacrylamide Gel Electrophoresis. PAGE under nondenaturing conditions was performed in Tris–glycine buffer, pH 8.3, using 30 mA/slab constant current at 4 °C. The acrylamide concentration was 11% (acrylamide–Bis 37.5:1 ratio).

SDS–PAGE was performed using 11 or 18% gel (acrylamide–Bis 37.5:1 or 90:1 ratio, for 11 or 18% acrylamide gel, respectively) in a buffer containing 0.1% SDS. Samples were solubilized with Laemmli sample buffer (50 mM Tris–glycine, pH 6.8, containing 0.1% SDS, 0.2% β-mercaptoethanol, and 30% glycerol) supplemented with bromophenol blue [5 μL of 0.02% (w/v) solution in water] and boiled for 3 min. The run was performed at 30 mA/slab in constant current at room temperature.

Heparin and Poly (Glu-Tyr) 4:1–Sepharose. Heparin and poly(Glu-Tyr) 4:1 were coupled with CNBr-activated Sepharose 4B according to a previously described procedure (Cuatrecasas, 1980).

RESULTS

Purification Procedure. Table I outlines the procedure followed to purify the kinase. In most of the cases the final kinase preparation shows a single protein band migrating at 67 kDa by SDS–PAGE (Figure 1). Only single band preparations are indicated in the present report as purified enzyme. The amount of the purified enzyme is very low (~0.2 μg of 67 kDa material from 100 g of calf uterus). The activity of the purified enzyme is stable for 3 days. Before the calmodulin–Sepharose chromatography, the enzymic activity in much more labile.

Interaction of the Enzyme with [(Fluorosulfonyl)benzoyl]-adenosine. FSO_4-Bz-Ado is a synthetic ATP analog which forms an inactive hydrolyzable binary complex with the catalytic site of kinases (Wyatt & Colman, 1977). Preincubation with this compound inhibited the ability of the uterus enzyme eluted from calmodulin–Sepharose to reactivate the phosphatase-inactivated estradiol receptor. The inhibition was dependent on the FSO_4-Bz-Ado concentration (panel A of Figure 2).

An aliquot of the same kinase preparation used in the experiment shown in panel A was incubated up to 60 min with [^{14}C]FSO_4-Bz-Ado. Samples were withdrawn at different times, concentrated by acid precipitation, analyzed by SDS–PAGE, stained by silver nitrate (panel B1 of Figure 2), destained, and fluorographed (panel B2 of Figure 2). The fluorogram shows only one ^{14}C band migrating as a 67-kDa protein. The specificity of the kinase interaction with [^{14}C]-FSO_4-Bz-Ado is evident from the disappearance of the ^{14}C 67-kDa protein band when the incubation was performed with an excess of a nonhydrolyzable ATP analog, the AMP-PNP (not shown).

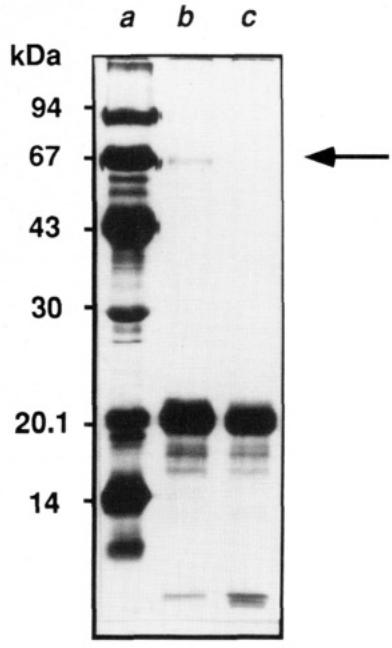

FIGURE 1: SDS–PAGE of the purified kinase. The kinase was lyophilized in the presence of purified soybean trypsin inhibitor as described in the section Reversible Denaturation of the Kinase. A sample containing trypsin inhibitor but no enzyme was submitted to the same procedure. Proteins were analyzed by 11% SDS–PAGE and stained by silver nitrate. Lane b is the sample containing the kinase and the carrier; lane c is the sample containing the carrier but no kinase. The arrow indicates the position of the kinase. Protein standards were coelectrophoresed (lane a).

Analysis of the Uterus Enzyme under Native and Denaturating Conditions. The purified enzyme submitted to PAGE under nondenaturing conditions showed a single enzymic activity peak (panel A of Figure 3) coincident with a single protein band seen in the lane stained by silver nitrate (Figure 3, top of panel A).

When the purified kinase was analyzed by SDS–PAGE a 67-kDa protein was detected by silver nitrate (Figure 1). The renaturation of a sample simultaneously analyzed by SDS–PAGE showed a peak of activity migrating as a 67-kDa protein (panel B of Figure 3). Therefore, enzymic activity activating the hormone binding of the receptor is associated with the 67-kDa protein.

The purified enzyme centrifuged through a sucrose gradient migrated as a single, sharp peak of activity corresponding to a protein with a 6 S sedimentation coefficient (panel A of Figure 4). The Stokes radius value evaluated by comparison with reference proteins and averaged from three different G-200 Sephadex chromatographies was 46 Å (panel B of Figure 4). This value was used together with the sedimentation coefficient value to calculate the molecular weight of the enzyme according to Siegel and Monti (1966). This was 114 kDa. When the molecular weight was calculated using the Andrews plot (Andrews, 1964), exclusively based on the behavior of the enzyme during the Sephadex G-200 chromatography, a value of 136 kDa was obtained. Therefore, the values of the molecular weight of the native enzyme range from 114 to 136 kDa.

Interaction of the Enzyme with [^{125}I] Calmodulin. Figure 5 shows the behavior of [^{125}I]calmodulin preincubated with the enzyme eluted from calmodulin–Sepharose and then submitted to centrifugation through a sucrose gradient. In the presence of EGTA, only the 1.9S peak of the free [^{125}I]-calmodulin was observed; in the presence of Ca^{2+}, the height of the 1.9S peak was reduced and a 6.3S peak of [^{125}I]-calmodulin appeared. Since the enzyme sediments at ~6 S (see panel A of Figure 4), apparently the enzyme forms a binary complex with calmodulin. This complex requires Ca^{2+}, since in the presence of EGTA the 6.3S peak was not observed.

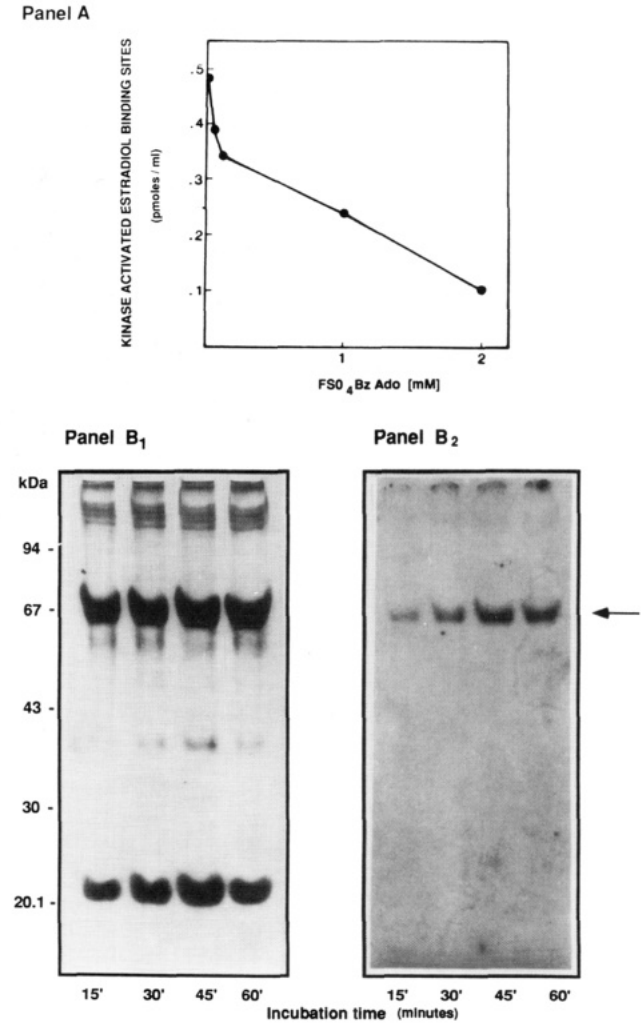

FIGURE 2: Inhibition of the kinase by 5′-[(fluorosulfonyl)benzoyl]-adenosine and fluorography with [^{14}C]FSO_4-Bz-Ado. (A) A 250-μL aliquot of the kinase eluted from calmodulin–Sepharose was incubated at 30 °C for 30 min with the indicated concentrations of FSO_4-Bz-Ado. The enzymic activity was assayed as described under Materials and Methods. (B1) A 300-μL aliquot of the kinase from calmodulin–Sepharose was supplemented with 100 μM [^{14}C]FSO_4-Bz-Ado, 10 mM Na_2MoO_4, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 10 μg/mL calmodulin and incubated at 30 °C for the indicated times. Proteins were precipitated by TCA using 10 μg/mL myoglobin as a carrier, analyzed by 11% SDS–PAGE, and then stained by silver nitrate. (B2) Fluorography of the destained gel presented in (B1). The arrow indicates the 67-kDa band.

Substrates of the Purified Kinase

Uterus Estradiol Receptor. Fifty percent of hormone binding activity of purified [^{3}H]estradiol–calf uterus receptor complex was inactivated by incubation with the uterus phosphatase. The mixture was divided in two aliquots and incubated with the purified kinase in the presence of either radioinert ATP to measure the reactivation, which was found to be complete, or [γ-^{32}P]ATP. Estradiol receptor was immunoprecipitated and its phosphorylation analyzed by autoradiography of SDS–PAGE. In Figure 6, lane 1 of panel A1 shows that the enzyme has phosphorylated the 67-kDa receptor, a 48-kDa protein which is probably a proteolytic product of the receptor (Katzenellenbogen et al., 1983; Van Osbree et al., 1984; Walter et al., 1985), and in addition a

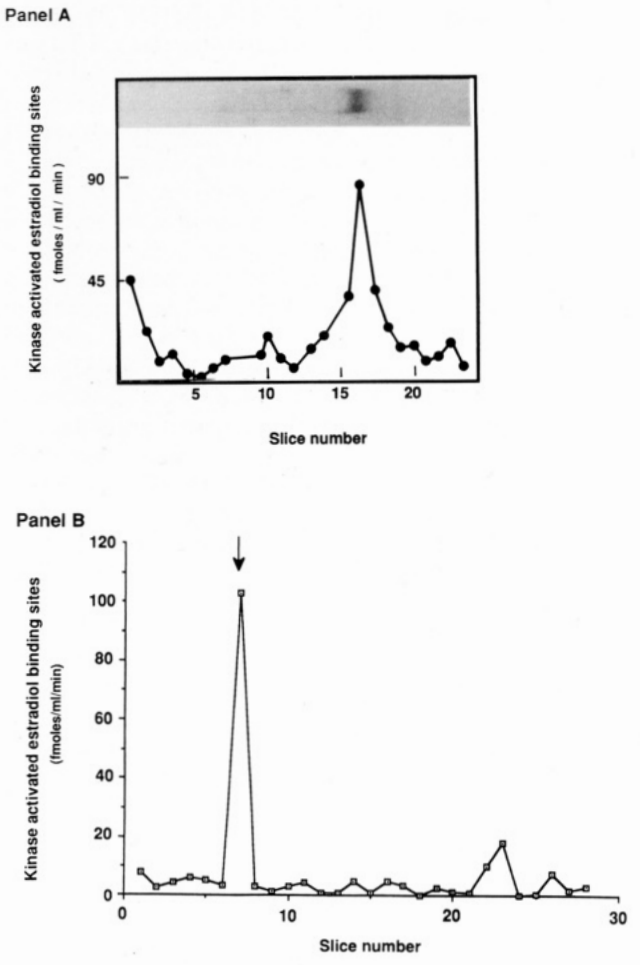

FIGURE 3: Polyacrylamide gel electrophoresis of the purified kinase under nondenaturating conditions and denaturating conditions followed by renaturation of the enzyme. (A) Two kinase samples (100 μL each) were submitted to 11% PAGE. One lane was stained with silver nitrate (see the top of the panel). The other lane was sliced into 3-mm fractions, and proteins were extracted with TGD buffer (300 μL). After centrifugation the enzymatic activity from each sample was assayed as described under Materials and Methods except for omitting the sample incubation in the absence of ATP. The kinase-activated binding sites were evaluated from the difference in hormone binding sites of each sample and a sample of identical composition except for substitution of the gel slice extract with TGD buffer. (B) Reversible denaturation of the kinase was performed as described under Materials and Methods using 11% SDS–PAGE. Protein standards were coelectrophoresed. The arrow shows the bovine serum albumin position (67 kDa).

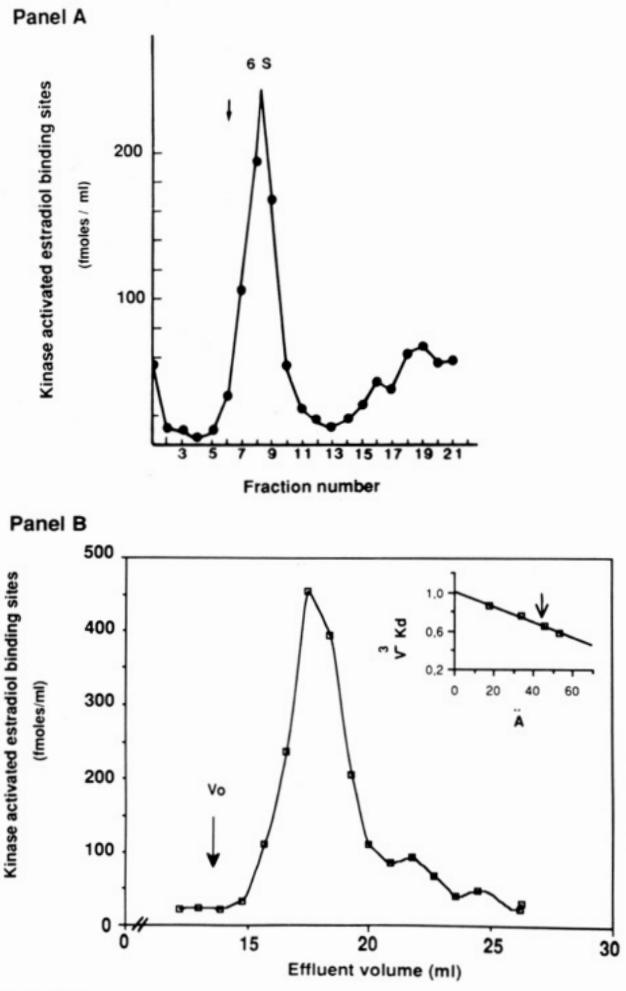

FIGURE 4: Centrifugation through sucrose gradient and G-200 gel filtration of the purified kinase. (A) The kinase (300 μL) was layered on top of a 10–30% sucrose linear gradient in TGD buffer (4 mL) and centrifuged at 105000g for 16 h at 2 °C in a SW 60 Ti rotor using a L8-55 Beckman ultracentrifuge. The gradient was collected in 200-μL fractions. The kinase activity was assayed as described under Materials and Methods. The arrow indicates the migration of serum bovine albumin (4.5 S). (B) The kinase (600 μL) was poured on a Sephadex-G200 column (15 × 180 mm) preequilibrated with TGD buffer and eluted at 8 mL/h flow rate. The void volume of the column was assessed by dextran T 2000. The eluate was collected in 940-μL fractions. Aliquots (250 μL) from each fraction were assayed for kinase activity as described under Materials and Methods except for omitting the samples incubated without ATP. The kinase-activated hormone binding sites were evaluated from the difference in binding activity of each sample and a sample of identical composition except for the substitution of the fraction aliquots with TGD buffer. The inset shows the calibration plot of the chromatography obtained using catalase, aldolase, albumin, and cytochrome c as standards. The known Stokes radii of the standard proteins are plotted versus their $K_{d3/1}$ experimentally determined (Porath, 1963). The arrow indicates the Stokes radius of the kinase derived from the $K_{d1/3}$ value (46 Å).

17-kDa protein. This protein might be either receptor-associated calmodulin or a proteolytic product of the receptor. When both, reactivation and ^{32}P phosphorylation of the receptor, were performed in the absence of free Ca^{2+}, only 5% of the inactivated sites were reactivated by the kinase and phosphorylation of the receptor was undetectable (lane 2 of panel A1).

Phosphoamino acid analysis of the ^{32}P 67-kDa protein eluted from gel lane 1 shown in panel A1 detected [^{32}P]tyrosine (panel A2 of Figure 6). Neither an increase of hormone binding nor ^{32}P labeling of the receptor was seen when the receptor, which had not been previously inactivated by the nuclear phosphatase, was incubated with the kinase (not shown).

Calmodulin. ^{32}P phosphorylation of calmodulin by the kinase was investigated. The autoradiography of SDS–PAGE showed that the highest ^{32}P incorporation into calmodulin occurred in the presence of purified kinase, purified estradiol–receptor complex, and Ca^{2+} (lane 5 of panel B1, Figure 6).

In the absence of Ca^{2+}, phosphorylation of calmodulin was still detected although at a much lower level (lane 4 of panel B1). This is consistent with the findings shown in panel A1 of this figure. Omission of either enzyme or estradiol–receptor complex from the incubation mixture abolished calmodulin phosphorylation (lanes 1 and 2 of panel B1, respectively). A control sample incubated under the same conditions as lane 5, except for the absence of calmodulin, was run in parallel (lane 3, panel B1). No ^{32}P 17-kDa protein was detected. This finding showed that the 17-kDa phosphoprotein seen in lanes 4 and 5 was calmodulin. Amino acid analysis of [^{32}P]-calmodulin eluted from lane 5 of panel B1 revealed [^{32}P]-tyrosine (panel B2 of Figure 6).

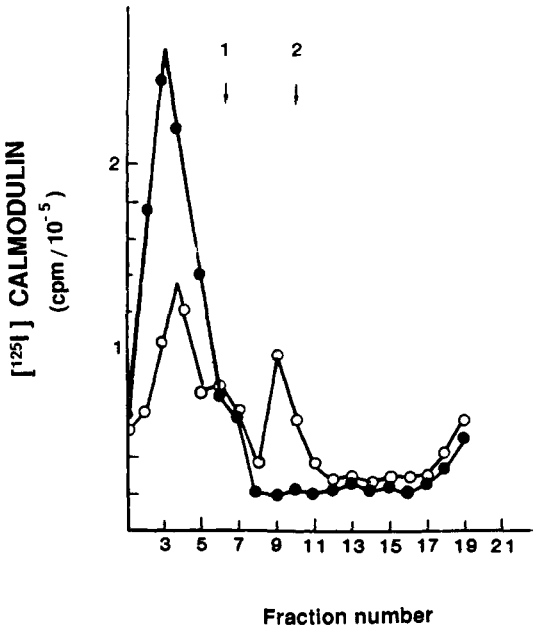

FIGURE 5: Effect of Ca^{2+} on interaction of [^{125}I]calmodulin with the kinase followed by sucrose gradient. The kinase eluted from calmodulin–Sepharose (100 μL) was incubated for 2 h at 0 °C with 30 μg of radioinert calmodulin and 1.5 μg of [^{125}I]calmodulin (specific activity 5 μCi/μg) in TD buffer containing 0.2 M KCl and 1 mM $CaCl_2$. A parallel sample was incubated under the same conditions except for substituting 1 mM $CaCl_2$ with 0.2 mM EGTA. The two samples were poured on top of two 4-mL linear 5–20% sucrose concentration gradients in TGD buffer supplemented with 0.2 M KCl, 0.8 μM [^{125}I]calmodulin (specific activity 5 μCi/μg), and either 1 mM $CaCl_2$ (open circles) or 0.2 mM EGTA (closed circles), respectively. Gradients were run in a SW 60 Beckman rotor at 105000*g* for 14 h and then fractionated into 200-μL aliquots; each aliquot was counted for ^{125}I radioactivity. The arrows indicate the migration of bovine serum albumin (4.5 S) and ovalbumin (3.5 S), respectively.

Histone H_2B. Phosphorylation of histone H_2B by the uterus kinase was also investigated (panel C of Figure 6). Lane 5 of (C) shows that the highest ^{32}P labeling of histone H_2B occurred when incubation was performed in the presence of enzyme, estradiol–receptor complex, and Ca^{2+}-calmodulin. Absence of either enzyme or estradiol–receptor complex completely abolished ^{32}P phosphorylation of histone H_2B even in the presence of Ca^{2+}-calmodulin (lanes 1 and 2 of panel C, respectively). In addition, Ca^{2+}-calmodulin enhanced phosphorylation of histone H_2B when assayed in the presence of estradiol–receptor complex (compare lanes 4 and 5 of panel C). ^{32}P 14-kDa protein was not seen when the substrate was omitted from the incubation mixture (lane 3 of panel C) or when the substrate alone was incubated with [γ-^{32}P]ATP (not shown).

Actin. Western blot analysis with anti-phosphotyrosine antibodies was used to analyze actin phosphorylation on tyrosine by the kinase. It was mostly observed in the presence of estradiol–receptor complex and Ca^{2+}-calmodulin (lane 3 of panel D, Figure 6). The absence of Ca^{2+} drastically reduced actin phosphorylation (lane 4 of panel D). In addition, removal of estradiol–receptor complex or kinase from the incubation mixture completely abolished actin phosphorylation even in the presence of Ca^{2+}-calmodulin (lanes 1 and 2 of panel D, respectively). The specificity of actin phosphotyrosyl residue interaction with antiphosphotyrosine antibodies was verified by the absence of such an interaction in the presence of phenylphosphate (not shown).

Mutants of the Synthetic Estradiol Receptor HEO. The HBD of the human estradiol receptor contains five tyrosine residues at positions 328, 331, 459, 526, and 537 (Walter et al., 1985; Green et al., 1986). Each tyrosine of the HBD was separately substituted with phenylalanine using site-directed mutagenesis (Wallace et al., 1981; Lathe, 1985). HEO and the five HE-F mutants were used to assay the kinase-induced activation of hormone binding and receptor ^{32}P phosphorylation. In the absence of kinase, only 4% of the HEO synthesized in the lysate reticulocyte binds hormone (panel A of Figure 7). Similar basal levels of hormone binding (ranging from 1 to 9%) were observed with the mutants. The kinase increased the binding of HEO and four mutants to ~50% of the synthetic receptors. Only the receptor mutated at position 537 showed no change in the basal binding level (panel A of Figure 7). Analysis of the ^{32}P-phosphorylated and immunoprecipitated estradiol receptor showed that all the receptors were phosphorylated by the kinase except the one mutated at position 537 (panel B2 of Figure 7). In addition to the ^{32}P 67-kDa receptor, other [^{32}P]proteins have been immunoprecipitated, including a 90-kDa protein. Parallel aliquots of rabbit reticulocyte lysate containing ^{35}S-labeled HEO receptor as well as its mutants were also submitted to the immunoprecipitation procedure described for the ^{32}P-phosphorylated samples (panel B1 of Figure 7). Each receptor was precipitated with similar efficiency by JS 34/32 antibody. Therefore, the absence of the phosphorylated HE 537 receptor in panel B2 is not due to inability of the antibody to precipitate this mutant.

Phosphorylation of Synthetic Peptides. The kinase phosphorylates the peptide K13E corresponding to 11 of 13 amino acids surrounding tyrosine 537 of the human estradiol receptor in the presence of estradiol–receptor complex; absence of Ca^{2+}-calmodulin strongly reduces the enzymic activity whereas in the absence of estradiol–receptor complex, even in the presence of Ca^{2+}-calmodulin, the activity is not detectable (Table II). Therefore, this peptide behaves like the protein substrates of the kinase. Both $E_{11}G_1$ peptide and angiotensin II are not substrates of the kinase under the conditions used to phosphorylate the K13E peptide.

DISCUSSION

The calf uterus enzyme studied in this paper was first identified and partially purified in our laboratory (Auricchio et al., 1981a, 1987a; Migliaccio et al., 1984). An enzyme with similar properties has been found in immature lamb uterus (Lahooti et al., 1990). We now report both extensive purification and characterization of the enzyme from calf uterus.

Three affinity chromatographies have been added to the initial purification procedure (Auricchio et al., 1981a): calmodulin–Sepharose, used on the basis of the ability of this tyrosine kinase to interact with calmodulin; poly(Glu-Tyr) 4:1–Sepharose, based on the property of most tyrosine kinases to phosphorylate tyrosyl residues near acidic amino acids (Neil et al., 1981; Patschinsky & Sefton, 1981; Smart et al., 1981; Hunter, 1982; Patschinsky et al., 1982; Casneille et al., 1982; Braun et al., 1984), and ATP–agarose, which interacts with nucleotide binding proteins including kinases (Lee & Johansson, 1975; Lee et al., 1977, 1978).

Inhibition of the enzymic activation of estradiol receptor binding sites by the ATP analog FSO_4-Bz-Ado together with labeling of the enzyme by the [^{14}C]ATP analog does indeed support the notion that the enzyme which confers hormone binding to the receptor is a kinase. This is strongly corroborated by the association of two properties, binding activation and receptor phosphorylation on tyrosine, with the purified enzyme preparation. Tyrosine phosphorylation of LHRH

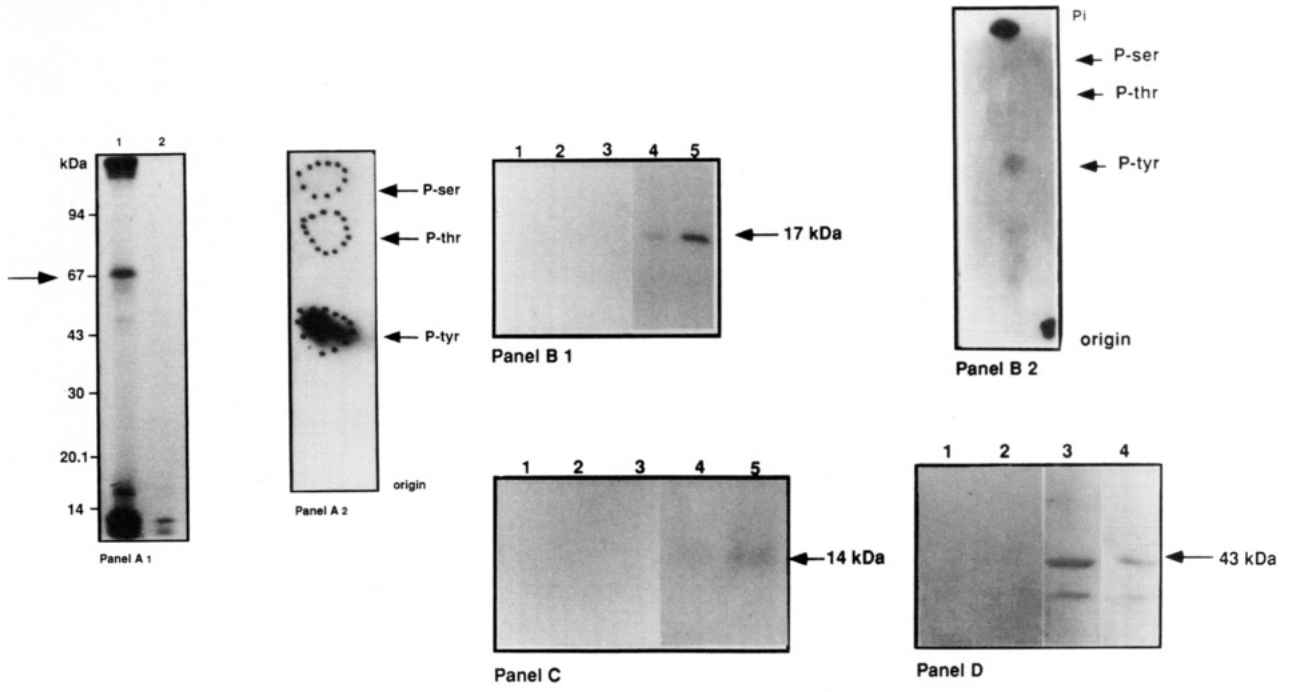

FIGURE 6: Phosphorylation of proteins by the purified tyrosine kinase. (A1 and A2) ^{32}P phosphorylation and phosphoamino acid analysis of purified estradiol receptor: (A1) Dephosphorylated estradiol receptor was incubated with the kinase as described under Materials and Methods. ^{32}P-Phosphorylated proteins were immunoprecipitated with antireceptor antibodies (JS 34/32) and analyzed by 11% SDS–PAGE. The gel was autoradiographed. Lane 1 is the autoradiography of the sample incubated in the absence of EGTA. Lane 2 is the autoradiography of the sample incubated in presence of EGTA. The arrow indicates the 67-kDa estradiol receptor position. (A2) The ^{32}P 67-kDa protein in lane 1 of (A1) was extracted from the gel and submitted to the phosphoamino acid analysis. (B1 and B2) ^{32}P phosphorylation and phosphoamino acid analysis of calmodulin: (B1) The ATP-free kinase was incubated with calmodulin. [^{32}P]Proteins were submitted to 18% SDS–PAGE, and the gel was autoradiographed. Lane 5 is the autoradiography of the sample incubated in the presence of calmodulin, the kinase, [^{3}H]-estradiol–receptor complex, and Ca^{2+}. Lane 4 is the sample incubated as in lane 5 except for the absence of Ca^{2+}. Lanes 1–3 are the autoradiographs of the control samples incubated as in lane 5 except for the absence of kinase (lane 1), [^{3}H]estradiol–receptor complex (lane 2), or calmodulin (lane 3). The arrow indicates the calmodulin position. (B2) The ^{32}P 17-kDa protein in lane 5 of (B1) was extracted from the gel and submitted to phosphoamino acid analysis. (C) ^{32}P phosphorylation of histone H_2B. The ATP-free kinase was incubated with histone H_2B. [^{32}P]Proteins were submitted to 18% SDS–PAGE, and the gel was autoradiographed. Lane 5 is the histone H_2B incubated in the presence of the kinase, [^{3}H]estradiol–receptor complex, and Ca^{2+}-calmodulin. Lane 4 is the sample incubated under the conditions of the sample in lane 5 except for the absence of Ca^{2+}-calmodulin. Lanes 1–3 are control samples incubated as in lane 5 except for the absence of kinase (lane 1), [^{3}H]estradiol–receptor complex (lane 2), or histone H_2B (lane 3). The arrow indicates the histone H_2B position. (D) Phosphorylation of actin followed by Western blot with anti-phosphotyrosine antibodies. Actin phosphorylated by the kinase was analyzed by 11% SDS–PAGE and then transferred to nitrocellulose. The membrane was hybridized with monoclonal anti-phosphotyrosine antibodies. Lane 3 is the sample of actin incubated with the kinase, [^{3}H]estradiol–receptor complex, Ca^{2+}, and calmodulin. Lanes 1, 2, and 4 are control samples incubated under the same conditions as the sample in lane 3 except for the absence of Ca^{2+} (lane 4), [^{3}H]estradiol–receptor complex (lane 1), or kinase (lane 2). The arrow indicates the actin position.

receptor also confers hormone binding (Liebow et al., 1991), suggesting that dependence of ligand binding upon tyrosine phosphorylation might be a mechanism shared by intracellular as well as membrane receptors.

Three different findings show that the enzyme migrates in SDS–PAGE as a 67-kDa protein. SDS–PAGE of the enzyme eluted from calmodulin–Sepharose preincubated with the ATP analog [^{14}C]FSO_4-Bz-Ado shows a single ^{14}C 67-kDa protein. A 67-kDa band is detected after silver nitrate staining of the purified enzyme submitted to SDS–PAGE. Finally, a reversible denaturation procedure allows the enzymic activity which confers hormone binding to the estradiol receptor to be localized in a 67-kDa protein. Analysis of the enzyme under nondenaturating conditions indicates a molecular weight between 114 and 136 kDa and suggests that the enzyme is a dimer of the 67-kDa protein. We have previously reported that the partially purified receptor in complex with estradiol stimulates tyrosine phosphorylation of the nonphosphorylated receptor by the kinase. This suggested that the estradiol–receptor complex stimulates the uterus kinase activity in vivo and consequently phosphorylation of different substrates of the kinase, including the nonphosphorylated, nonhormone binding receptor (Auricchio et al., 1987b). This hypothesis is now reinforced by the present finding that actin, calmodulin, and histone H_2B are indeed substrates of the purified kinase and their phosphorylation is also stimulated by estradiol–receptor complex. Nonhormone binding estradiol receptor in tamoxifen-treated MCF-7 cells accumulates (Leclerq et al., 1982). This could be explained by inhibition in these cells of tyrosine kinase similar or identical to the one characterized in the present paper. In fact the anti-estrogen tamoxifen reduces the ability of estradiol to stimulate the kinase-dependent activation of estradiol binding to the receptor (Auricchio et al., 1987b). Since we have used purified enzyme, purified estradiol–receptor complex, and purified substrates, it appears that a direct interaction between the complex and the kinase occurs, so activating the kinase. Dimeric structure of the tyrosine kinase could explain why estradiol is required for the kinase activation. In the presence of estradiol, the 67-kDa receptor dimerizes (Fawell et al., 1990; Forman & Samuels, 1990). Dimerization of the receptor may be required for efficient interaction with the enzyme, which is apparently a dimer of a 67-kDa protein.

Ca^{2+}-calmodulin not only stimulates phosphorylation of nonhormone binding receptor but also phosphorylation of actin and histone H_2B. The use of these substrates has shown that Ca^{2+}-calmodulin activates the kinase only in the presence of estradiol–receptor complex. This suggests that calmodulin

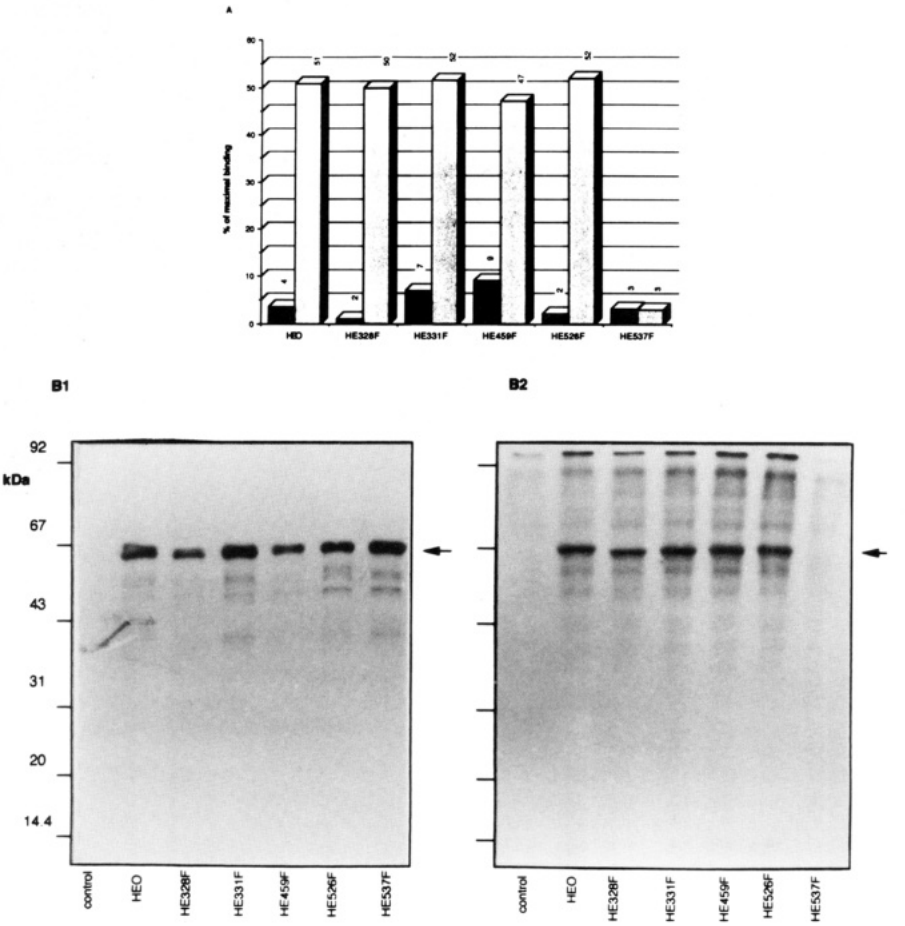

FIGURE 7: In vitro activation and ^{32}P phosphorylation of HEO human estradiol receptor and HBD tyrosine–phenylalanine mutants by the uterus kinase. HEO mRNA and HE 328-537F mutant mRNAs were translated in rabbit reticulocyte lysate and used. (A) Hormone binding activation by the uterus kinase was followed as described under Materials and Methods. The columns indicate the percent of maximal binding (1 mol of bound hormone/mol of synthesized receptor) of HEO and its mutants. Closed bars: samples incubated in the absence of ATP. Open bars: samples incubated in the presence of ATP. (B1) Fluorography of [^{35}S]methionine-labeled proteins synthesized in lysate in the absence (control) or in the presence of HEO mRNA or mutant HE 328-537F mRNA. Aliquots of lysate (5 μL) were diluted to 200 μL with TGD buffer. All the samples were immunoprecipitated with antireceptor JS 34/32 antibodies and then submitted to 11% SDS–PAGE. (B2) HEO and HE 328-537F mutants were ^{32}P phosphorylated by the uterus kinase, immunoprecipitated with JS 34/32 antibodies, and submitted to 11% SDS–PAGE. The gel was autoradiographed. A control (lysate not containing receptor mRNA) was run in parallel. The arrow indicates the 67-kDa estradiol receptor position. Receptor synthesized in 10 μL of lysate: HEO 78, HE328F 77, HE331F 66, HE459F 72, HE526F 77, and HE537F 69 fmol.

activates the kinase through interaction with the estradiol–receptor complex rather than through the kinase. Ca^{2+}-dependent interaction between calmodulin and estradiol receptor has previously been seen in vitro (Castoria et al., 1988) and recently confirmed (Bouhoute & Leclercq, 1992). Alternatively, it is possible that calmodulin activates the enzyme by interacting with it only after estradiol–receptor complex association with the kinase.

Calmodulin is also a substrate of the uterus tyrosine kinase. Its phosphorylation requires estradiol–receptor complex and is stimulated by Ca^{2+}. Tyrosine phosphorylation of calmodulin by insulin receptor kinase and $pp60^{c\text{-}src}$ has been described (Fukami & Lipmann, 1985; Graves et al., 1986; Fukami et al., 1986; Sacks et al., 1988; Wong et al., 1988). Tyrosine phosphorylation of calmodulin might affect its interaction with other proteins (Williams et al., 1991).

Here we show that actin, a major protein component of microfilaments and a variety of nonmuscle cellular types, and calmodulin, a cytoskeleton-associated protein, are phosphorylated on tyrosine by the purified tyrosine kinase. This suggests that estradiol may regulate the cytoskeleton architecture through tyrosine phosphorylation of these two proteins. Phosphorylation on tyrosine of actin might create specific binding sites for the actin binding proteins having regions of similarity to the SH-3 domain (Drubin et al., 1990).

HEO differs from HEGO human synthetic receptor with valine in place of glycine at position 400 (Tora et al., 1989). Its ability to bind estradiol when compared with the wild-type recombinant receptor HEGO is altered at 25 °C but not at 4 °C, the temperature we use for the in vitro estradiol binding assay. Only a small amount of both receptors synthesized in reticulocyte lysate binds hormone (Kumar et al., 1986; Tora et al., 1989; Migliaccio et al., 1991). The uterus enzyme phosphorylates both receptors and confers hormone binding to a large amount of them (Migliaccio et al., 1989, 1991). The kinase phosphorylates exclusively the HBD on tyrosine and confers hormone binding to it (Migliaccio et al., 1989). Five point mutated HEO receptors, each with a different tyrosine of the HBD replaced by phenylalanine, were translated in vitro and incubated with the kinase. Only the mutant with phenylalanine at position 537 in place of tyrosine was neither ^{32}P phosphorylated nor acquired hormone binding. This shows that phosphorylation of this tyrosine residue is necessary for the kinase-induced hormone binding activation. It does not exclude that additional tyrosine residues of the receptor must be phosphorylated in a progressive and orderly way (Levine et al., 1991) for the hormone binding acquisition. On the

Table II: Phosphorylation of the Peptide K13E by the Purified Kinase[a]

Peptide K13E	
Lys*Lys*Lys*ValValProLeuTyrAspLeuLeuLeuGlu*	
Peptide E11G1	Angiotensin II
GluAspAlaGluTyrAlaAlaArgArgArgGly	AspArgValTyrIleHisProPhe

Peptide K13E	
conditions	cpm/50 μL
standard	2152
without Ca^{2+}-calmodulin	319
without estradiol receptor	−100
without kinase	50

[a] In the upper portion of the table, the peptide K13E sequence is reported. Italicized amino acids correspond to those surrounding tyrosine 537 of the human estradiol receptor. The sequences of two peptides, peptide E11G1 and angiotensin II, which are not phosphorylated by the kinase, are also shown. The results of peptide K13E phosphorylation have been averaged from three different experiments. Standard conditions indicate a sample containing Ca^{2+}-calmodulin, purified calf uterus estradiol–receptor complex, and kinase. Lack of phosphorylation of peptides E11G1 and angiotensin II was confirmed by autoradiography of high-voltage paper electrophoresis of the samples.

carboxyl side of tyrosine 537, aspartic and glutamic acids are spaced by three leucines. In contrast with several phosphorylation site motifs of known tyrosine kinases (Kemp & Pearson, 1990), no acidic amino acid is present close to tyrosine 537 on its amino side. A synthetic peptide corresponding to 11 out of 13 amino acids surrounding tyrosine 537 can be phosphorylated by the kinase. In contrast, peptide $E_{11}G_1$ and angiotensin II are not phosphorylated. $E_{11}G_1$ with an amino acid sequence similar to the major phosphorylation site of $pp60^{src}$ (Swarup et al., 1983, 1984) and angiotensin II only have acidic amino acids close to the amino side of tyrosine. These findings suggest that the kinase phosphorylates tyrosines with acidic amino acids close to the carboxyl side. There are acidic amino acids close to the carboxyl side of the two tyrosine residues of calmodulin in addition to those close to the amino side of tyrosine (Klee et al., 1980). Actin contains three tyrosine residues presenting acidic amino acids close to the carboxyl side of tyrosine and five residues with acidic amino acids close to both sides of tyrosine (Vanderkerckhove & Weber, 1979). Histone H_2B does not have acidic amino acids close to the carboxyl side of tyrosines. It has a cluster of three tyrosines at positions 37, 40, and 42 and an aspartic acid in position 51 (Iwai et al., 1972; Ohe et al., 1979). This motif may explain the weak phosphorylation of histone H_2B by the kinase.

The finding of tyrosine phosphorylation at position 537 on the human estradiol receptor calls for further comments. Tyrosine 537 is close to cysteine 530, which is the covalent binding site of affinity-labeling estrogen and anti-estrogen in the human estradiol receptor (Harlow et al., 1989). In addition, amino acids around cysteine 530 discriminate between estrogens and anti-estrogens (Pakdel & Katzenellenbogen, 1992). Mouse estrogen deletion mutant 121-507 does not bind hormone, whereas mutant 121-538 does (Fawell et al., 1989). Furthermore, tyrosine 537 is in the initial portion of exon 8. This is a region conserved in the estradiol receptor and divergent from the other nuclear receptors. It is therefore important in specific hormone binding (Ponglikitmongkol et al., 1988). It is of interest that while substitution of tyrosine 537 with phenylalanine abolishes binding due to receptor phosphorylation it does not affect the small basal hormone binding of the receptor. This suggests that a portion of the estradiol receptor can also bind hormone without tyrosine phosphorylation (Auricchio, 1989).

In conclusion, the present findings show that estradiol is able in vitro to stimulate the tyrosine phosphorylation of different proteins and suggest that this hormone stimulates tyrosine phosphorylation in target cells. Such a stimulation could have a role in cell duplication induced by estradiol. In fact, increased tyrosine phosphorylation has frequently been associated with cell multiplication induced by growth factors as well as by retroviruses (Hunter & Sefton, 1980, 1985; Bishop, 1985). In our laboratory a study on the ability of estradiol to stimulate tyrosine phosphorylation in intact cells shows that this steroid in complex with its receptor stimulates tyrosine kinases (manuscript submitted).

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