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Progesterone receptor subunits are high-affinity substrates for phosphorylation by epidermal growth factor receptor

(phosphotyrosine receptor kinase/steroid receptor)

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ABSTRACT **Purified preparations of epidermal growth factor (EGF) receptor were used to test hen oviduct progesterone receptor subunits as substrates for phosphorylation catalyzed by EGF receptor. Both the 80-kilodalton (kDa) (A) and the 105-kDa (B) progesterone receptor subunits were phosphorylated in a reaction that required EGF and EGF receptor. No phosphorylation of progesterone receptor subunits was observed in the absence of EGF receptor, even when Ca^{2+} was substituted for Mg^{2+} and Mn^{2+}. Phospho amino acid analysis revealed phosphorylation at tyrosine residues, with no phosphorylation detectable at serine or threonine residues. Two-dimensional maps of phosphopeptides generated from phosphorylated 80- or 105-kDa subunits by tryptic digestion revealed similar patterns, with resolution of two major, several minor, and a number of very minor phosphopeptides. The K_m of progesterone receptor for phosphorylation by EGF-activated EGF receptor was 100 nM and the V_{max} was 2.5 nmol/min per mg of EGF receptor protein at 0°C. The stoichiometry of phosphorylation/hormone binding for progesterone receptor subunits was 0.31 at ice-bath temperature and approximately 1.0 at 22°C.**

Tyrosine phosphorylation has become recognized as a protein modification reaction with potential roles in regulation of cell metabolism, reproduction, and differentiation. Viral transforming gene products and their normal cell homologues (1–4) and receptors for epidermal growth factor (EGF) (5), platelet-derived growth factor (6–8), and insulin (9–10) all catalyze transfer of phosphate from ATP to tyrosine residues of proteins. The best-characterized substrates for these kinases are the kinase proteins themselves; all undergo autophosphorylation. In addition, viral transformation of cells or binding of ligands to specific cell surface receptors stimulates tyrosine phosphorylation of cellular proteins (11–13). With the exception of vinculin (14), these cellular substrates of tyrosine kinases have been characterized only by their migration in one- or two-dimensional gel electrophoresis systems.

Preliminary studies in this laboratory have indicated that only a few proteins in crude extracts of normal tissues became phosphorylated in an EGF-dependent manner by purified EGF receptor preparations (15). Additionally, a major human placental protein substrate of EGF receptor kinase exhibited properties reminiscent of steroid hormone receptors, including high molecular weight, ability to bind to DNA-cellulose, and dual localization in nuclear and cytoplasmic fractions (unpublished data). Phosphorylation of steroid receptors has been implicated in regulation of hormone binding (16, 17) and transformation of steroid receptors to the nuclear binding state (17). Moreover, phosphorylation of steroid receptors at serine has been demonstrated *in vitro* (18) and in receptors isolated from $^{32}P_i$-labeled tissues (19) or cultured cells (20). To test for possible involvement of tyrosine phosphorylation in regulation of steroid receptor function, we examined purified hen oviduct progesterone receptor as a substrate for purified EGF receptor kinase.

EXPERIMENTAL PROCEDURES

Purification of Progesterone Receptor. The transformed progesterone receptor was purified from hen oviducts by a procedure similar to that of Chang *et al.* (21), to be described in detail elsewhere. The final steps in the procedure were affinity chromatography on *N*-(12-aminododecyl)-3-keto-4-androsten-17β-carboxamide-Sepharose (22) and chromatography on phosphocellulose. Purification of approximately 20,000-fold was achieved from the starting cytosol with an overall yield of 10–20% and a specific activity of 0.2–0.8 mol of [^{3}H]progesterone bound per mol of receptor A (80-kDa) and B (105-kDa) subunits (kDa, kilodaltons). Sodium dodecyl sulfate/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) analysis of purified preparations showed minor impurities migrating at 280, 150, and 45 kDa on gels overloaded for receptor protein. In addition, there were faint bands immediately below the 80- and 105-kDa receptor bands that could arise from limited proteolysis; these increased when the serine protease inhibitor phenylmethylsulfonyl fluoride was omitted during receptor preparation. Receptor preparations were concentrated at the final step by precipitation with $(NH_4)_2SO_4$ and dissolved in, and dialyzed against, 20 mM Hepes, pH 7.4.

Photoaffinity labeling of the receptor subunits with ^{3}H-labeled 17α,21-dimethyl-19-norpregn-4,9-diene-3,20-dione ([^{3}H]R5020) was performed after affinity chromatography. Bound [^{3}H]progesterone was displaced with $HgCl_2$ (23, 24), and after separation of the progesterone aporeceptor from free hormone and reactivation of hormone binding with dithiothreitol, [^{3}H]R5020 was added for binding overnight at 4°C. The [^{3}H]R5020–receptor complex was then purified by chromatography on phosphocellulose, irradiated with a xenon lamp through a Pyrex filter (25), and dissolved in electrophoresis sample buffer after precipitation with acetone.

Purification of EGF Receptor. EGF receptor was purified over 6000-fold and at >80% yield from Triton X-100 extracts of human epidermoid A431 cells by affinity chromatography on Fractogel TSK-immobilized ricin-binding subunit, followed by affinity chromatography on EGF-Fractogel. The purified receptor protein migrated as a single 160-kDa band in $NaDodSO_4$/PAGE and 1 mg of receptor protein bound not less than 5 nmol of ^{125}I-labeled EGF at saturation and 22°C.

PAGE. Phosphoproteins were resolved by $NaDodSO_4$/PAGE on 1.5-mm polyacrylamide gel slabs by the method of

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Abbreviations: EGF, epidermal growth factor; kDa, kilodalton(s); $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; R5020, 17α,21-dimethyl-19-norpregn-4,9-diene-3,20-dione.

Laemmli (26). The gels were stained with Coomassie blue and bands containing ^{32}P were detected by autoradiography on Kodak X-Omat x-ray film with a DuPont Lightning-Plus intensifying screen.

Phosphopeptide Mapping. Gel sections containing ^{32}P-labeled bands were mashed and the labeled proteins were eluted during an overnight incubation in an aqueous solution of 50 mM NH_4HCO_3, 0.1% $NaDodSO_4$, 5% (vol/vol) 2-mercaptoethanol, and bovine serum albumin at 50 μg/ml. This procedure achieves a 70–80% yield with no detectable degradation of phosphorylated proteins. Eluted proteins were precipitated with 20% trichloroacetic acid and the precipitated proteins were washed twice with acetone, dissolved in performic acid, and incubated on ice for 2 hr. Samples were then lyophilized and digested with 30 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin in 500 μl of 50 mM NH_4HCO_3 for 18 hr at 23°C and for a further 4 hr at the same temperature after the further addition of 20 μg of TPCK-treated trypsin. Soluble peptides were separated by centrifugation, lyophilized, dissolved in 10 μl of 1% $(NH_4)_2CO_3$, and applied onto 10 × 10 cm cellulose plates. Electrophoresis was carried out in the first dimension in 1% $(NH_4)_2CO_3$ at 400 V for 45 min, followed by chromatography in the second dimension in 1-butanol/pyridine/acetic acid/water (15:10:3:12, vol/vol) (14).

Phospho Amino Acid Analysis. Radiolabeled proteins were resolved by gel electrophoresis, eluted from the gel, and hydrolyzed in 6 M HCl at 108°C for 2 hr. After addition of phosphotyrosine, phosphoserine, and phosphothreonine reference standards, the hydrolysate was fractionated by electrophoresis at pH 1.9 (2.5 hr at 400 V) and at pH 3.5 (2 hr at 400 V) and analyzed for phosphorylated amino acids (27). Reference standards were visualized by staining with ninhydrin.

Analytical Methods. Protein was determined by the dye-binding assay of Bradford (28), using bovine albumin as standard. Tritium was measured by liquid scintillation spectrometry as described (23), and ^{32}P was quantified by Cerenkov radiation in a Beckman liquid scintillation counter.

Materials. Laying hen oviducts were obtained from El Monte Rabbit (S. El Monte, CA). A431 cells were obtained from G. Todaro and J. DeLarco (National Cancer Institute). EGF was isolated as described (29). [^{3}H]Progesterone (55 Ci/mmol) and [γ-^{32}P]ATP (>2 kCi/mmol) were from Amersham; [^{3}H]R5020 (87 Ci/mmol) was from New England Nuclear (1 Ci = 37 GBq). Biochemicals and protein standards for gel electrophoresis were obtained from Sigma, and reagents for gel electrophoresis were from Bio-Rad. Fractogel TSK was obtained from Pierce.

RESULTS

EGF Receptors Catalyze Phosphorylation of Progesterone Receptor Subunits. Purified progesterone receptor preparations were tested for phosphate acceptor activity in a system containing purified EGF receptor kinase. When EGF receptor was tested alone in the radiophosphorylation system, 160-kDa EGF receptor protein was the sole labeled product and its radiophosphorylation was reduced when EGF was omitted (Fig. 1, lane 1 vs. lane 2). When purified progesterone receptor was added to this system, EGF stimulated phosphorylation of the 80-kDa (A) and 105-kDa (B) progesterone receptor subunits (lane 3 vs. lane 4). EGF-stimulated phosphorylation of progesterone receptor subunits by EGF receptor kinase also was observed when a partially purified progesterone receptor preparation (approximately 5% pure after the affinity chromatography step) was tested (lane 5 vs. lane 6).

No endogenous progesterone receptor kinase activity was observed in the purified progesterone receptor preparation. Phosphorylation of the 80- and 105-kDa bands in the standard reaction mixture (Fig. 1) had an absolute requirement for EGF receptor (data not shown). No Ca^{2+}-activated autophosphorylation of progesterone receptor subunits (31) was observed when Ca^{2+} was the sole divalent cation in the system described for Fig. 1.

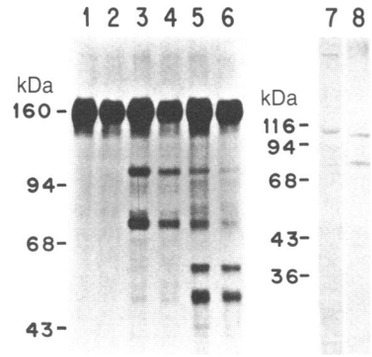

FIG. 1. EGF- and EGF receptor-dependent phosphorylation of progesterone receptor subunits in purified and partially purified preparations. The reaction systems contained purified EGF receptor (1.6 pmol of EGF binding capacity), 20 mM Hepes buffer at pH 7.4, 20 μM [γ-^{32}P]ATP (500–1000 cpm/pmol), 4.0 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.2% Triton X-100 in a final volume of 50 μl. EGF was present at 50 ng (lanes 1, 3, and 5), progesterone receptor purified through phosphocellulose chromatography at 3.4 μg (lanes 3 and 4), and progesterone receptor purified through steroid affinity chromatography at 20 μg (lanes 5 and 6). Purified EGF receptor was incubated first in the presence or absence of EGF for 3 min at 30°C in 20 μl of 20 mM Hepes buffer. Reaction vessels were chilled on ice; progesterone receptor preparations were then added in 10 μl, and reactions were initiated with 20 μl of solution containing divalent cations and labeled ATP. After incubation for 15 min at 0°C, reactions were terminated with 12 μl of 5× concentrated electrophoresis sample buffer. Proteins were resolved by $NaDodSO_4$/PAGE and visualized by autoradiography. Reference samples of purified progesterone receptor were resolved by $NaDodSO_4$/PAGE and visualized by staining with Coomassie blue (lane 7) or by fluorography (30) after photoaffinity labeling with [^{3}H]R5020 (lane 8).

The procedure employed for purification of hen oviduct progesterone receptor yielded a preparation containing intact 80- and 105-kDa A and B receptor subunits (Fig. 1, lane 7). These were in the "transformed" state, as indicated by their ability to bind to phosphocellulose. The major protein bands present in the preparation (lane 7) corresponded to the migration of the synthetic progestin [^{3}H]R5020 that was attached covalently by UV irradiation (Fig. 1, lane 8). Liquid scintillation counting analysis of these bands revealed equal amounts of label in the 80- and 105-kDa subunits.

Incorporation of $^{32}P_i$ into each major phosphoprotein band of Fig. 1 is summarized in Table 1. Autophosphorylation of 160-kDa EGF receptor was stimulated over 2-fold by EGF (lane 1 vs. lane 2). When EGF receptor autophosphorylation and substrate phosphorylation were examined under conditions that favored determination of initial phosphorylation rates rather than maximal phosphate acceptor capacities,

Table 1. Quantitation of EGF receptor autophosphorylation and phosphorylation of progesterone receptor subunits

	Protein phosphorylation, pmol per lane					
Band, kDa	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
160	10.1	5.0	10.2	5.1	8.0	4.0
105	—	—	1.9	0.8	1.0	0.5
80	—	—	2.2	0.9	1.1	0.5
60	—	—	—	—	1.1	1.1
50	—	—	—	—	1.3	1.3

Phosphoprotein bands from the gel shown as Fig. 1 were excised and radioactivity was determined by Cerenkov counting.

EGF stimulated phosphorylation of progesterone receptor and EGF receptor by at least 6-fold. Purified progesterone receptor did not reduce EGF receptor autophosphorylation (lanes 3 and 4). However, partially purified progesterone receptor (lanes 5 and 6) reduced phosphorylation in the 160-kDa band and produced phosphoprotein components migrating slightly ahead of the 160-kDa band. These findings may indicate the presence of protease or phosphotyrosine phosphatase contaminants in the progesterone receptor preparation at this purification stage.

At both stages of progesterone receptor purification, EGF stimulated phosphorylation of the 80- and 105-kDa subunits to the same extent as it stimulated EGF receptor phosphorylation. In contrast, $^{32}P_i$ incorporation into the two nonreceptor phosphoproteins migrating at 50 and 60 kDa was not stimulated upon EGF addition (Table 1, lanes 5 and 6). Phosphorylation of 50- and 60-kDa proteins did not require EGF receptor kinase. No reduction in their phosphorylation was observed when EGF receptor was omitted in an experiment otherwise identical to that shown in Fig. 1, lane 6.

Stoichiometry of Progesterone Receptor Subunit Phosphorylation. Phosphorylation of the 105- and 80-kDa progesterone receptor subunits increased linearly with increasing EGF receptor concentration up to 2.4 pmol of EGF binding capacity (Fig. 2). At this point, phosphorylation of progesterone receptor subunits was maximal, and 0.31 mol of phosphate was incorporated per mol of progesterone-binding activity. A 3- to 4-fold higher incorporation of phosphate was observed at 22°C.

Progesterone Receptor Subunits Are Phosphorylated at Tyrosine Residues. After phosphorylation catalyzed by EGF receptors, progesterone receptor subunits were resolved by $NaDodSO_4$/PAGE, hydrolyzed in acid, and analyzed for ^{32}P-labeled amino acids (Fig. 3 *A* and *B*). The only ^{32}P-labeled phospho amino acid detected was phosphotyrosine. Recovery of input radioactivity averaged 95%; 35%, 40%, and 20% of input radioactivity were recovered as phosphotyrosine, P_i, and undigested peptides, respectively. No radioactivity was detected in phosphoserine or phosphothreonine.

Progesterone Receptor Subunits Are Phosphorylated at Regions of Sequence Homology. Phosphorylated progesterone receptor subunits were analyzed by two-dimensional peptide mapping after extensive digestion with trypsin. Brief autoradiography revealed two major and five minor labeled peptides for each subunit (Fig. 4 *A* and *B*), and a mixture of the labeled phosphopeptides from the 80- and 105-kDa subunits

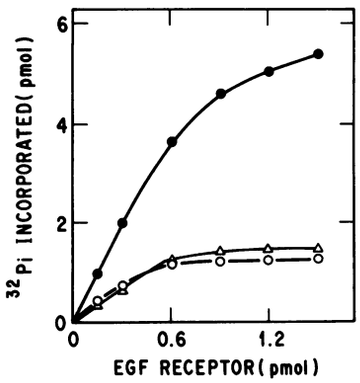

FIG. 2. Phosphate acceptor capacity of progesterone receptor subunits. Phosphorylation of purified progesterone receptor (3.4 μg containing 9.0 pmol of hormone-binding capacity) catalyzed by various amounts of EGF receptor (in units of EGF-binding capacity) was performed as described for Fig. 1. Incorporation of ^{32}P into 160-kDa (●), 105-kDa (○), and 80-kDa (△) protein bands was determined as described for Table 1.

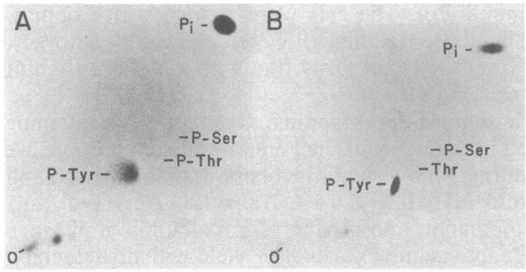

FIG. 3. Analysis of phosphorylated amino acids in progesterone receptor subunits after incubation with EGF receptor kinase. Phosphorylation and resolution of progesterone receptor subunits by $NaDodSO_4$/PAGE was performed as in Fig. 1, lane 3. Progesterone receptor subunits were eluted from gels and hydrolyzed with 6 M HCl at 108°C for 2 hr. Phosphorylated amino acids were separated in two dimensions as described in *Experimental Procedures*. Radioactive regions were identified by autoradiography of thin-layer plates and analyzed by Cerenkov counting. The radioactive regions near the origin (o) are from undigested peptides; P-Ser, phosphoserine; P-Tyr, phosphotyrosine; P-Thr, phosphothreonine. (*A*) Phospho amino acid analysis of 80-kDa phosphoproteins. (*B*) Phospho amino acid analysis of 105-kDa phosphoprotein.

produced a pattern nearly identical to that obtained with either subunit alone (Fig. 4*C*). Two-thirds of the applied radioactivity was recovered in the seven most prominent phosphopeptides, 62 and 20% of which was in the two most highly labeled ones. Prolonged autoradiography revealed more than 10 very minor phosphorylated peptides (Fig. 4 *D* and *E*); many of these were present in both the 105-kDa and the 80-kDa progesterone receptor subunits (Fig. 4*F*). Thus, EGF receptor tyrosine kinase phosphorylates progesterone receptor 80- and 105-kDa subunits primarily in regions of sequence homology.

Progesterone Receptor Subunits Are High-Affinity Substrates for Phosphorylation. The affinity of progesterone receptor subunits for EGF receptor kinase was determined by measuring phosphorylation rates at different concentrations

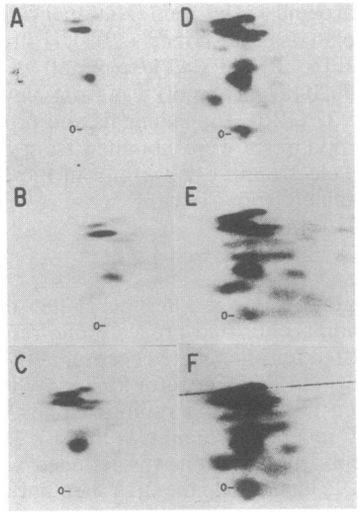

FIG. 4. Tryptic phosphopeptide mapping of progesterone receptor subunits. Phosphorylation and gel electrophoretic resolution of progesterone receptor subunits was performed as described for Fig. 1, lane 3. The 105- and 80-kDa subunits were eluted from the gel and digested with trypsin and the fragments were analyzed by peptide mapping. (*A* and *D*) Maps of fragments from 80-kDa subunit; (*B* and *E*) maps of fragments from 105-kDa subunit; (*C* and *F*) maps of 1:1 mixture of fragments from 80-kDa plus 105-kDa subunits. (*A–C*) Brief autoradiography; (*D–F*) prolonged autoradiography. Radioactive regions were scraped from the plates and analyzed by Cerenkov counting.

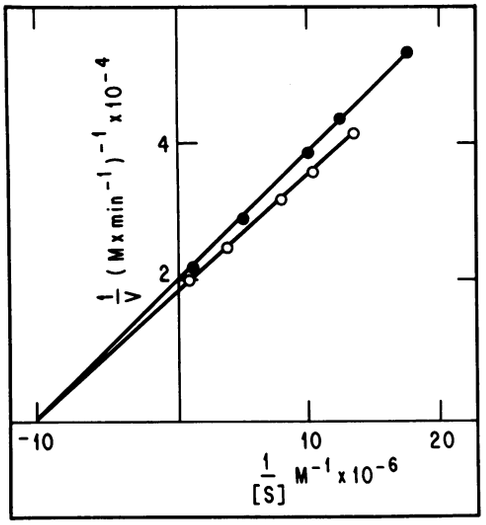

FIG. 5. Lineweaver–Burk plot for phosphorylation of progesterone receptor subunits by EGF receptor kinase. Various concentrations of purified progesterone receptor substrate were phosphorylated in reactions catalyzed by 0.3 pmol of EGF-binding capacity of EGF receptor in the 50-μl system described for Fig. 1. Progesterone receptor subunits were resolved by NaDodSO$_4$/PAGE and the bands were visualized by autoradiography and cut from the gel for analysis by Cerenkov counting. ○, 80-kDa subunit; ●, 105-kDa subunit.

of these phosphate acceptor proteins. Double-reciprocal plots (Fig. 5) yielded similar K_m (90–100 nM) and V_{max} (2.5 nmol of phosphate incorporated per min per mg of EGF receptor protein) values for both subunits.

DISCUSSION

The recently discovered phenomenon of increased protein phosphorylation on tyrosine residues in cells transformed by tumor viruses (1–4) or stimulated by hormones such as EGF (11, 32) has directed much effort towards identifying substrates for both viral- and cellular-gene derived tyrosine kinases. The criteria for identifying these substrates include: (*i*) differential labeling in $^{32}P_i$-labeled cells in response to hormone or a shift from nonpermissive to permissive condition for expression of transformed phenotype, (*ii*) the presence of tyrosine residues in sequences similar to those in described substrates for tyrosine kinases, (*iii*) substrate activity with tyrosine kinases *in vitro*, and (*iv*) a defined regulatory function for a candidate substrate. The progesterone receptor satisfies several of these criteria. Its subunits are the highest affinity substrates yet described for a tyrosine kinase by several orders of magnitude. Moreover, steroid receptors are known regulatory proteins that act at nuclear sites (33, 34).

Table 2 summarizes the K_m value for known EGF receptor kinase substrates. The affinity for progesterone receptor subunits (0.0001 mM) is several orders of magnitude greater than any other values reported. Synthetic peptides that are identical to or resemble in their sequence a tyrosine-containing phosphate acceptor region on *src* protein have K_m values ranging from 0.2 to 6.0 mM. Several hormones that have homology to this region are higher-affinity substrates (K_m = 0.05–1.30 mM) but have affinity less than 1/500th of that of progesterone receptor subunits. The only substrate that compares favorably is a 94-kDa protein that is the major EGF receptor kinase substrate in human placental extracts (unpublished data), but the function of that protein has not yet been identified.

Progesterone receptor subunits can be digested with trypsin to demonstrate at least seven independent phosphate-acceptor sites, of which two are major sites. The remarkable property of these sites is their uniform presence in both receptor subunits. This contrasts to a relative lack of homology between the A and B progesterone receptor subunits for regions containing tyrosine residues in general. Maps of peptides from progesterone receptor subunits labeled with ^{125}I by Chloramine-T-mediated iodination revealed homology in only 4 of nearly 50 peptides resolved after proteolytic digestion (45). This striking conservation of tyrosine kinase substrate regions in both subunits suggests possible roles for these regions. Additionally, the multiple sites for tyrosine phosphorylation could provide opportunities for differential access to different species of tyrosine kinase. This property could allow individualistic responses to different cellular tyrosine kinases.

The substrate activity of progesterone receptors for EGF receptor kinase provides a mechanism by which peptide hormones, including growth factors, could influence steroid hormone action at the level of a steroid receptor protein.

Table 2. Substrates for EGF receptor tyrosine kinase

Substrate	K_m, mM	Ref.
Naturally occurring proteins		
Progesterone receptor	0.0001	This report
Placental 94-kDa substrate	0.0001	Unpublished
Gastrin-17	0.05	35
Human growth hormone	ND	36
Anti-pp60*src* IgG	ND	37, 38
Middle T tumor antigen	ND	39
34-kDa *src* kinase substrate	ND	15
Tubulin	ND	40
Synthetic peptides		
[Val^5]Angiotensin II	1.30	41
Leu-Asp-Thr-Thr-Gly-Gln-Glu-Glu-*Tyr*-Ser-Ala	0.243	42
Arg-Arg-Leu-Ile-Glu-Asp-Asn-Glu-*Tyr*-Thr-Ala-Arg-Gly	0.28	43
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Tyr*-Ala-Ala-Arg-Gly	0.50	43
Ile-Glu-Asp-Asn-Glu-*Tyr*-Thr-Ala-Arg-Gln-Gly	1.1	43
Arg-Arg-Leu-Ile-Ala-Asp-Ala-Glu-*Tyr*-Ala-Ala-Arg-Gly	1.1	43
Leu-Glu-Asp-Ala-Glu-*Tyr*-Ala-Ala-Arg-Arg-Arg-Gly	1.5	44
Arg-Arg-Leu-Ile-Glu-Ala-Ala-Glu-*Tyr*-Ala-Ala-Arg-Gly	1.8	35
Leu-Arg-Arg-Ala-*Tyr*-Leu-Gly	6.0	35

ND, not determined.

Synergy between EGF and glucocorticoids has been reported (46); EGF and dexamethasone combined have a greater mitogenic potency than the sum of the potencies of both acting singly. The possible biological significance of our results can be tested in cells containing both EGF and progesterone receptors (47, 48).

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