Calcineurin-Mediated Dephosphorylation of the Human Placental Membrane Receptor for Epidermal Growth Factor Urogastrone†

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ABSTRACT: The findings of our work were 2-fold: (1) calcineurin (from bovine brain) can catalyze the complete dephosphorylation of the phosphotyrosine and phosphoserine residues in the human placental receptor for epidermal growth factor urogastrone (EGF-URO), and (2) the major calmodulin-binding protein of human placental membranes is a calcineurin-related protein. In terms of its metal ion dependence ($Ni^{2+} > Mn^{2+} > Co^{2+}$), its calmodulin dependence, and its sensitivity to inhibitors (Zn^{2+}, fluoride, orthovanadate), the phosphotyrosyl protein phosphatase activity of calcineurin, using the EGF-URO receptor as substrate, paralleled the enzyme activity measured with *p*-nitrophenyl phosphate (PNPP) as a substrate. These characteristics distinguish calcineurin from other classes of protein phosphotyrosyl phosphatases. Calcineurin purified from placental membranes was similar to, if not identical with, bovine brain calcineurin in terms of enzymatic specific activity toward PNPP, subunit electrophoretic mobilities, and immunological cross-reactivity. The enzymatic properties and comparative abundance of calcineurin in the placenta membranes suggest that this enzyme may play an important role in regulating the phosphorylation state of those receptors (e.g., for EGF-URO or insulin) also known to be present in the membranes.

Calcineurin has recently been demonstrated to possess calmodulin (CaM)[1]-regulated phosphatase activity toward a wide variety of phosphoseryl proteins (Stewart et al., 1982; Blumenthal & Krebs, 1983; King et al., 1984; Gupta et al., 1984). In addition, calcineurin can also dephosphorylate nonprotein substrates like phosphoenolpyruvate, *p*-nitrophenyl phosphate (PNPP), and free phosphotyrosine (Wang et al., 1984; Pallen & Wang, 1983). In view of the ability of phosphotyrosine to serve as a substrate, we have begun to evaluate phosphotyrosine-containing cellular proteins as potential substrates for calcineurin.

The epidermal growth factor urogastrone (EGF-URO) receptor of A431 carcinoma cells and of human placenta exhibits EGF-URO-stimulated tyrosine kinase activity both in terms of autophosphorylation and in terms of the phosphorylation of a variety of protein and polypeptide substrates (Carpenter et al., 1978; Cohen et al., 1980; Pike et al., 1984). We have used human placental EGF-URO receptor, phosphorylated at tyrosine and serine residues, as a substrate for bovine brain calcineurin. In this paper, we describe the dephosphorylation of the phosphoserine and phosphotyrosine residues of the receptor by calcineurin and the existence of calcineurin in placental membrane.

Materials and Methods

Materials. Calcineurin and calmodulin were purified from bovine brain as previously described (Sharma et al., 1983). EGF-URO was prepared from mouse submaxillary glands as described (Savage & Cohen, 1972). Monoclonal antibodies against the α and β subunits of calcineurin were prepared as previously described (Matsui et al., 1985). Formaldehyde-fixed *Staphylococcus aureus* bacteria were provided by Dr. P. Lee (The University of Calgary, Canada). We are grateful to Dr. M. D. Waterfield (Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, England) and to Dr. R. C. Richards (University of Liverpool, England) for making available to us the monoclonal anti-EGF-URO receptor antibody (EGFR1; Waterfield et al., 1982).

Membrane Preparation, Membrane Phosphorylation, and Immunoprecipitation of Receptor. Crude microsomal membrane preparations of human placenta were made from full-term tissue obtained at Caesarean section as described previously (Hock & Hollenberg, 1980) except that buffers contained 5 mM EDTA and EGTA to minimize proteolysis. Aliquotted sample was stored at −70 °C for further use.

Membrane Phosphorylation. Membrane phosphorylation was done at 0 °C in a reaction containing 20 mM Hepes (pH 7.4), 2 mM $CaCl_2$, 20 mM $MgCl_2$, and 50 μM Na_3VO_4 with placental membrane (0.75 mg/mL) which had been incubated at 0 °C for 10 min with 200 ng/mL EGF-URO. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (15 μM, 10 Ci/mmol) and terminated by the addition of an equal reaction volume of solubilization buffer (20 mM Hepes, pH 8.0, 2 mM EGTA, 50 μM Na_3VO_4, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 2% Trasylol).

After incubation in solubilization buffer for 60 min on ice, the phosphorylated sample was clarified by centrifugation (60

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[1] Abbreviations: CaM, calmodulin; CaN, calcineurin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGFR1, monoclonal anti-EGF-URO receptor antibody; EGF-URO, epidermal growth factor urogastrone; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PNPP, *p*-nitrophenyl phosphate; Na_3VO_4, sodium orthovanadate; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Cl_3CCOOH, trichloroacetic acid; Trasylol, aprotinin; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s).

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min, 100000g). A 300-μL aliquot of the supernatant was mixed with 500 μL of a 1:100 dilution of a stock EGFR1 antibody-containing ascites fluid (0.19 mg of protein/mL) in phosphate-buffered saline and incubated for 18 h at 4 °C. Two hundred microliters of a 10% suspension of washed, formaldehyde-fixed *Staphylococcus aureus* bacteria was then added and incubated for 60 min at 4 °C. The immunoprecipitate was collected by centrifugation, washed 3 times with 1 mL of wash buffer [10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, and 0.1% SDS], and then resuspended in 300 μL of 20 mM Hepes buffer (pH 7.4).

Phosphoamino Acid Analysis. The phosphorylated EGF-URO receptor, eluted from SDS–PAGE gel pieces overnight by using an Isco Model 1750 sample concentrator (1 W, 4 °C), was precipitated by 10% Cl_3CCOOH on ice, and the pellet was washed once with acetone. Protein hydrolysis and phosphoamino acid analysis were performed as described (Hunter & Sefton, 1980).

Calcineurin-phosphatase Assays. Calcineurin (80 μg/mL) was incubated with 1 mM metal ion plus or minus 100 μg/mL calmodulin at 25 °C for 60 min prior to its addition to immunoprecipitated phosphorylated receptor or to PNPP. Reactions were done at 30 °C in 20 mM Hepes (pH 7.6). Receptor dephosphorylation reactions contained receptor (2000–4000 cpm), 250 μM metal ion, 20 μg/mL calcineurin plus or minus 25 μg/mL calmodulin, and other additions as indicated. Dephosphorylation was monitored by laser densitometric scans of autoradiograms of SDS–PAGE-resolved receptor. The PNPP hydrolysis reactions contained 2 mM PNPP, 3 μg/mL calcineurin plus or minus 3.75 μg/mL calmodulin, and 1.04 mM Ni^{2+}. Dephosphorylation of PNPP was monitored spectrophotometrically as described previously (Pallen & Wang, 1983).

Partial Purification of Calcineurin from Placental Membranes. Membranes were prepared as outlined above but with PMSF (0.2 mg/mL), soybean trypsin inhibitor (5 μg/mL), and benzamidine (0.2 mg/mL) added to all buffers. Membrane pellets were resuspended with a Potter–Elvehjem homogenizer in buffer A [20 mM Tris (pH 7.0), 1 mM $Mg(OAc)_2$, 1 mM imidazole, 10 mM 2-mercaptoethanol, 0.1 mM EGTA, and 10% glycerol] containing 1% Triton X-100, stirred at 4 °C for 90 min, and then centrifuged at 100000g for 60 min. The supernatant was diluted 100-fold with buffer A and applied to a DEAE-cellulose column (15 × 2.5 cm) equilibrated with buffer A. The column was washed several times with buffer A, and protein was eluted with buffer A containing 0.22 M NaCl. Eluted protein was pooled, and CaM-binding proteins were isolated by affinity chromatography on a CaM–Sepharose 4B column as described for bovine brain calcineurin (Sharma et al., 1983).

Immunoblot Detection of Calcineurin. Immunoblotting was performed essentially as described by Towbin et al. (1979). The blots were developed as described by Shalev et al. (1980).

Gel Electrophoresis and Autoradiography. SDS–PAGE (8.5% acrylamide) was performed according to the method of Laemmli (1970). Destained gels were dried and exposed at −70 °C to Kodak X-Omat R film using Du Pont Cronex Lightning-Plus intensifying screens.

Results

Phosphorylation of the EGF-URO Receptor and Phosphoamino Acid Analysis. Stimulation of the phosphorylation of the placental EGF-URO receptor in the presence of EGF-URO is illustrated in Figure 1. Immunoprecipitation with monoclonal anti-receptor antibody (Figure 1A, lane 3) confirmed that the 170000-kDa phosphorylated band observed

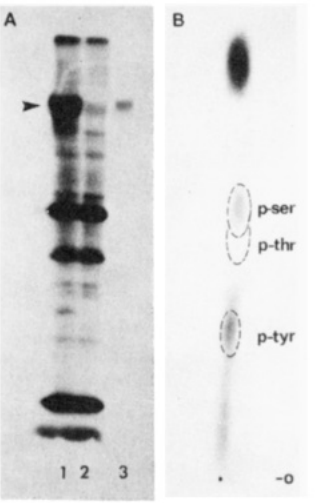

FIGURE 1: Phosphorylation, immunoprecipitation, and phosphoamino acid analysis of the EGF-URO receptor. (A) SDS–PAGE and autoradiography of phosphorylated membranes (lanes 1 and 2) and of immunoprecipitated receptor (lane 3). Phosphorylation reactions were done in the presence (lanes 1 and 3) or absence (lane 2) of EGF-URO. The arrow indicates the position of the EGF-URO receptor. (B) Autoradiography of thin-layer electrophoretic separation of an acid-hydrolyzed sample of the phospho-EGF-URO receptor. The areas enclosed by dashes represent the positions of standard phosphoamino acids as visualized with ninhydrin. Abbreviations: p-ser, phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine; o, origin.

Table I: Effects of Divalent Cations and Calmodulin on Calcineurin-Mediated Receptor Dephosphorylation[a]

additions	dephosphorylation (%)	additions	dephosphorylation (%)
Ni^{2+}, CaM	0	Co^{2+}, CaM	0
Ni^{2+}, CaN	76	Co^{2+}, CaN	40
Ni^{2+}, CaN, CaM	100	Co^{2+}, CaN, CaM	53
Mn^{2+}, CaM	0	Ca^{2+}, CaM	0
Mn^{2+}, CaN	33	Ca^{2+}, CaN	0
Mn^{2+}, CaN, CaM	64	Ca^{2+}, CaN, CaM	13

[a] Receptor dephosphorylation (15 min at 30 °C) was monitored by densitometric scanning of gel autoradiograms in which the radiodensities of control samples (receptor alone) were assigned a value of zero dephosphorylation. A value of 100% corresponds to an absence of radiodensity in the autoradiogram.

in other studies (Cohen et al., 1980; Das et al., 1977; Hock et al., 1980) corresponded to the receptor. Analysis of phosphoamino acid content of the radiolabeled receptor revealed the presence of phosphotyrosine and phosphoserine but no detectable phosphothreonine (Figure 1B). In preliminary experiments with crude membranes, it was possible to detect rapid dephosphorylation of the receptor (complete within 20 min) in the absence of phosphatase inhibitors. This activity was separated into at least two chromatographic fractions using PNPP as an indicator of tyrosine phosphatase activity. Because of the presence of heterogeneous membrane PNPP phosphatase activity which would have complicated studies of the action of pure calcineurin, receptor phosphorylated in intact membranes was first isolated by immunoadsorption for the kinetic experiments described below.

Characterization of Metal Ion Stimulated EGF-URO Receptor Phosphatase Activity. The well-characterized preparation of calcineurin from bovine brain was used for the majority of our work. The activity of calcineurin toward the immunoprecipitated phosphoreceptor was determined with various metal ions. As shown in Table I, calcineurin activity was highest in the presence of Ni^{2+} followed by Mn^{2+} and Co^{2+}; Ca^{2+} had no detectable effect. All detectable phos-

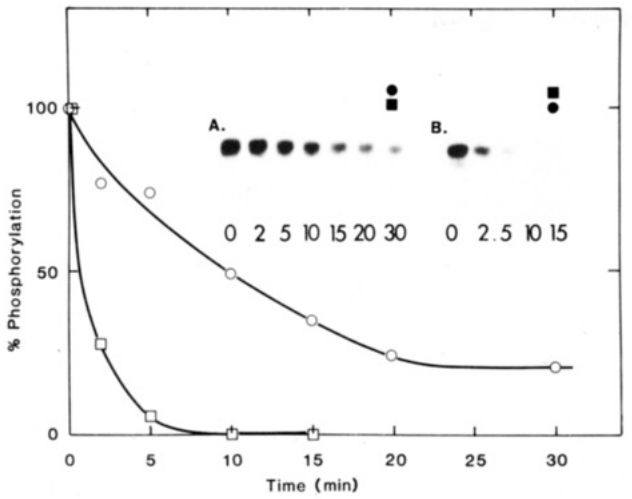

FIGURE 2: Time courses of EGF-URO receptor dephosphorylation by calcineurin. Calcineurin (CaN) was preincubated with Mn^{2+} (○) or Ni^{2+} (□) in the presence of calmodulin as described under Materials and Methods and was then added to immunoprecipitated, phosphorylated receptor to start the reaction. At timed intervals, an aliquot of the reaction mixture was analyzed by SDS–PAGE and autoradiography [insert: (A) Mn^{2+}-activated CaN; (B) Ni^{2+}-activated CaN]. Dephosphorylation rates were determined from densitometric scans of the autoradiograms, wherein the radiodensities of control samples (minus CaN) were assigned a value of 100%. (●, ■) Control samples without CaN at 20 and 30 min.

Table II: Effects of Various Compounds on Ni^{2+}-Activated Calcineurin-phosphatase Activity[a]

additions	concn (mM)	% inhibition	
		PNPP	phosphoreceptor
none		0	0
Zn^{2+}	0.2	83	100
	0.1	76	69
	0.05	64	31
vanadate	0.1	0	0
fluoride	10	20	28
	5	3	17
phosphoserine	10	70	99
phosphothreonine	10	35	20
phosphotyrosine	10	20	28
PNPP	5		40
	1		19

[a] Receptor dephosphorylation by Ni^{2+}/CaM-activated calcineurin (15 min at 30 °C) was monitored by densitometry of the autoradiograms in which the radiodensities for samples lacking inhibitors or calcineurin were taken as 0 or 100% inhibition, respectively. PNPP dephosphorylation was monitored spectrophotometrically.

phatase activity was stimulated further by CaM (Table I). The above results are in keeping with the observed efficacy of these metal ions to stimulate calcineurin-phosphatase activity toward PNPP (Pallen & Wang, 1984). The reaction catalyzed by Ni^{2+}-activated calcineurin was complete within 10 min (Figure 2), thus indicating that calcineurin could dephosphorylate both phosphoseryl and phosphotyrosyl residues in the receptor.

Effects of Phosphatase Inhibitors. Sodium orthovanadate (Swarup et al., 1982) and Zn^{2+} (Brautigan et al., 1981) have been reported to inhibit phosphotyrosine phosphatases. We found that vanadate did not inhibit Ni^{2+}-activated calcineurin activity toward either the EGF-URO receptor or PNPP (Table II). In contrast, the Mn^{2+}-activated calcineurin activity toward the receptor was partially inhibited by 25, 50, and 100 μM vanadate (19, 47, and 61%, respectively) (data not shown). Zinc, as opposed to vanadate, was able to inhibit Ni^{2+}-activated calcineurin activity toward either the EGF-URO receptor or PNPP (Table II).

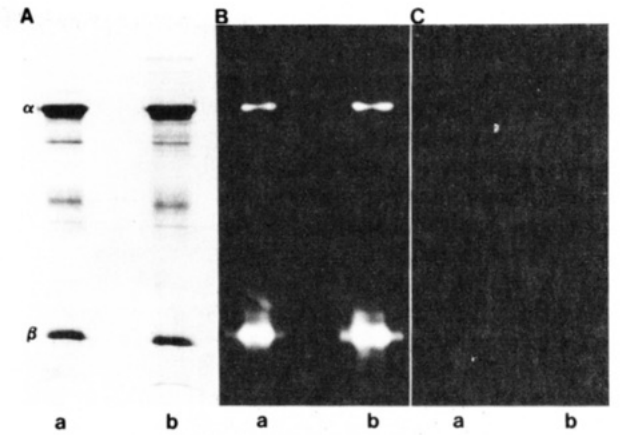

FIGURE 3: Comparison of bovine brain calcineurin and human placental membrane calmodulin-binding protein fraction by SDS–PAGE and immunoblotting. (A) Bovine brain calcineurin (lane a, 5 μg) and human placental calmodulin-binding proteins (lane b, 5 μg) were analyzed by SDS–PAGE (12% gels). The positions of the large and small subunits of bovine brain calcineurin are indicated by α and β, respectively. (B) Immunoblots of bovine brain calcineurin (lane a, 1 μg) and human placental membrane calmodulin-binding proteins (lane b, 1 μg) using anti-α and -β bovine brain calcineurin monoclonal antibodies. (C) As described for (B), but bovine brain calcineurin monoclonal antibodies were omitted from the immunoblotting procedure.

Sodium fluoride has been shown to inhibit phosphoseryl and phosphothreonyl protein phosphatase activities (Khatra & Soderling, 1978) but to have no effect on phosphotyrosyl protein phosphatase activity (Brautigan et al., 1981; Foulkes et al., 1983; Chernoff et al., 1984; Horlein et al., 1982; Gallis et al., 1981; Foulkes et al., 1981). Millimolar concentrations of sodium fluoride only partially inhibited the ability of Ni^{2+}-activated calcineurin to dephosphorylate either the receptor or PNPP (Table II).

In view of the relatively high activity of calcineurin toward free phosphotyrosine (Pallen & Wang, 1983), we examined the ability of phosphotyrosine, phosphothreonine, and phosphoserine to inhibit calcineurin-catalyzed receptor and PNPP dephosphorylation. At a concentration of 10 mM, phosphoserine, though not a substrate, was the most effective inhibitor (Table II). PNPP itself was also a good inhibitor of receptor dephosphorylation (Table II).

Partial Purification of Calcineurin from Placental Membranes. The CaM-binding protein fraction from solubilized placental membranes, representing approximately 0.3% of the original membrane protein, was examined for the possible existence of a calcineurin-like protein. Electrophoretic and Western blot analyses showed that monoclonal antibodies directed against either the α or the β subunit of bovine brain calcineurin cross-reacted with corresponding proteins isolated from placental tissue (Figure 3B). The SDS gel electrophoretic pattern of the placental membrane CaM-binding protein fraction was virtually identical with that of purified bovine brain calcineurin. The placental protein and purified bovine brain calcineurin exhibited similar Ni^{2+}- and CaM-stimulated specific activities using PNPP as a substrate [brain calcineurin, 1.5 ± 0.1 μmol min^{-1} mg^{-1} (mean ± SEM for five separate preparations); placental CaM-binding fraction, 1.5 μmol min^{-1} mg^{-1}].

Discussion

A major finding of our study was that calcineurin can catalyze the complete dephosphorylation of the placental membrane EGF-URO receptor, a protein that can contain both phosphotyrosyl and phosphoseryl residues in intact cells. Similarly, calcineurin can dephosphorylate the nonphysio-

logical substrate phosphotyrosylcasein (Chernoff et al., 1984). In terms of metal ion dependence, calmodulin dependence, and sensitivity to inhibitors, the action of calcineurin on the EGF-URO receptor parallels the action of the enzyme on PNPP (Pallen & Wang, 1983, 1984). Thus, most if not all of the properties of the enzyme as studied with PNPP as a substrate may apply in general to proteins containing phosphotyrosyl residues. As demonstrated for PNPP calcineurin-phosphatase activity, the activation of receptor calcineurin-phosphatase requires metal ions (Ni^{2+}, Mn^{2+}, or Co^{2+}). In addition, these metal ions can also interact with calmodulin to cause a further stimulation of enzyme activity (Pallen & Wang, 1984). Although Ca^{2+} cannot directly activate calcineurin, Ca^{2+}/CaM can further stimulate the activity of calcineurin which has been preactivated by Ni^{2+}, Mn^{2+}, or Co^{2+} (Pallen & Wang, 1984).

The phosphotyrosyl phosphatase activity of calcineurin appears distinct from three other groups of phosphotyrosine phosphatases identified to date. The phosphatases of one group are maximally active in the presence of EDTA and fluoride but are inhibited by micromolar concentrations of Zn^{2+} (Foulkes et al., 1983; Gallis et al., 1981; Chernoff & Li, 1983). A second group (group 2A), which exhibits phosphoseryl protein phosphatase activity, also possesses Mn^{2+}-stimulated, EDTA-inhibited phosphotyrosyl phosphatase activity (Foulkes et al., 1983); these enzymes are partially inhibited by fluoride. A third group of phosphotyrosyl phosphatases, more commonly known as alkaline phosphatases, is inhibited by EDTA but not by fluoride (Foulkes et al., 1983; Swarup et al., 1981). In terms of its activity toward the EGF-URO receptor, calcineurin appears to constitute a fourth class of phosphotyrosyl protein phosphatase(s). Like the first group, calcineurin is inhibited by micromolar concentrations of Zn^{2+}; like the type 2A protein phosphatases, calcineurin is inhibited by EDTA. In terms of its sensitivity to fluoride and its pH dependence, calcineurin is clearly distinct from the first group of phosphotyrosyl phosphatases and from alkaline phosphatases. In addition, calcineurin has CaM-stimulated phosphotyrosyl phosphatase activity. No other phosphotyrosyl phosphatase activities have yet been shown to be subject to protein–protein regulation.

The electrophoretic mobility of the placental calcineurin subunits, the cross-reactivity of the subunits with the anti-calcineurin monoclonal antibodies, and the Ni^{2+}- and CaM-stimulated PNPP phosphatase activity of the placental membrane CaM-binding fraction indicate a close relationship if not identity between placental calcineurin and bovine brain calcineurin. The occurrence of calcineurin in the placental membrane fraction is in accord with previous data pointing to the distribution of calcineurin between particulate and cytosolic fractions (Tallant et al., 1983; Aitken et al., 1984). Given a membrane-associated location, calcineurin, in conjunction with other membrane-associated phosphatases, could serve as an important regulator of the phosphorylation state of a variety of membrane constituents, including the receptors for insulin and EGF-URO.

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