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PROTEOLYSIS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR BY ENDOGENOUS CALCIUM-ACTIVATED NEUTRAL PROTEASE FROM RAT LIVER

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SUMMARY: In A-431 membranes but not in rat liver membranes, the epidermal growth factor (EGF) receptor was converted from a M_r=180,000 to a M_r=160,000 form by a protease activated when cells were broken in the presence of calcium. Calcium-activated neutral protease (CANP) activity in rat liver cytosol was separated from its protein inhibitor by DEAE-cellulose chromatography. When fractions containing this protease activity were incubated with rat liver membranes in the presence of calcium, the M_r=180,000 form of the receptor was converted to the M_r=160,000 form. This conversion was blocked both by the separated endogenous inhibitor and by leupeptin. Apparently CANP is a highly regulated endogenous protease which could degrade the EGF receptor-kinase in most tissues.

INTRODUCTION: The EGF receptor has recently been shown to be a protein kinase (1,2) whose activity is enhanced when EGF binds (3,4,5,6). This kinase catalyzes the phosphorylation of tyrosine residues (7) on itself (1) and other exogenous and endogenous substrates (1,3,4,5) in intact cells (8), isolated membranes (1,3,4,5,9), solubilized membranes (6) and in affinity purified receptor preparations (1,6). While the kinase activity of the EGF receptor is controlled by EGF levels outside the cell (8), another control on kinase activity may be limited proteolysis of the receptor (10-12). Limited proteolysis can convert the primarily autophosphorylating form of the EGF receptor-kinase (M_r=180,000), into a form (M_r=160,000) with greater activity toward exogenous substrates (1).

One protease that produces limited proteolysis of the EGF receptor-kinase is activated when cells are broken (10). This proteolytic activity has been suggested by us (11) and others (12) to be CANP based on requirements for calcium, pH optimum,

ABBREVIATIONS: EGF, epidermal growth factor; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)NN'-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; CANP, calcium-activated neutral protease.

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inhibition by leupeptin (11) and sulfhydryl sensitivity (12). However, the calcium-activated proteolysis of the EGF receptor-kinase has only been studied in some detail in cultured A-431 cells, a human epidermoid carcinoma cell line. We report here that the EGF receptor-kinase in a normal tissue, rat liver, is degraded by an endogenous CANP only after an endogenous inhibitor is removed. We suggest that CANP is a highly regulated cytoplasmic protease capable of degrading the EGF receptor-kinase in all tissues.

MATERIALS AND METHODS: Mouse EGF, which was provided by Dr. Stanley Cohen, Vanderbilt University, was prepared as described previously (13). The A-431 human epidermoid carcinoma cells were provided by Dr. J. DeLarco, National Institute of Health. Sprague-Dawley rats were purchased from Harlan Industries. Dulbecco's modified Eagle's medium, calf serum and penicillin-streptomycin were obtained from Grand Island Biological Company. New England Nuclear supplied the [γ-^{32}P] ATP at a specific activity of 3000 Ci/mmole and ^{125}I as iodide (carrier-free). SDS-PAGE protein standards for molecular weight determinations were purchased from Bio-Rad Laboratories. Synthetic leupeptin, bovine pancreas deoxyribonuclease I and casein (purified powder) were obtained from Sigma Chemical Company. All other reagents and chemicals were reagent grade and were purchased from common suppliers.

The A-431 cells were grown to confluency and a crude membrane fraction was prepared as described previously (11) by scraping the attached cells into a harvesting buffer (50 mM boric acid, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.2) according to the method of Thom et al (14).

Rat liver membranes were prepared according to Cuatrecasas (15) with the following modifications. Three-month old, male, Sprague-Dawley rats were sacrificed by decapitation and the liver removed, washed 2 times in isotonic saline at 0° C and weighed. Two 2 g portions of liver were each suspended in 20 ml of 0.25 M sucrose, 25 mM Tris-HCl, pH 7.4 at room temperature, at 0°C. Then one portion was made 0.1 mM in EGTA by adding 20 µl of 100 mM disodium EGTA, pH 7.4, while the other portion was made 1 mM in $CaCl_2$ by adding 200 µl of 100 mM $CaCl_2$. Following homogenization, the samples sat at room temperature for 10 min, were cooled to 0°C on ice and were centrifuged at 600xg for 10 min. The supernatants were collected and 200 µl of 100 mM disodium EGTA, pH 7.4 was added to the supernatant which contained the added calcium. The procedure of Cuatrecasas (15) was followed exactly from this point except that the membrane pellet was washed and finally resuspended in 20 mM HEPES, pH 7.4 and stored at -70°C.

The A-431 membrane preparations (30 µl) were phosphorylated in the presence of 1 mM Mn^{++} with [γ-^{32}P] ATP (3-6 µCi) in the presence or absence of EGF in a volume of 60 µl at 0°C and the reaction was terminated as described previously (11,16). Rat liver membranes were phosphorylated in the same way using 50 µg membrane protein per 60 µl reaction volume. Protein was determined according to Bradford (17) using bovine serum albumin as a standard. The molecular weights of the labeled proteins were determined by SDS-PAGE on 5% or 6% polyacrylamide gels followed by autoradiography of the stained, dried gels as described previously (16).

With the same rat livers used to prepare liver membranes, a CANP was purified and separated from its endogenous inhibitor by ion exchange chromatography on DEAE-cellulose as described by Waxman (18). The supernatant from a low ionic strength homogenate of 20 g of liver was mixed with 20 ml of settled DEAE-cellulose for one hour at 0°C. Then the resin was washed thoroughly with buffer A (10 mM Tris-HCl, 0.1 mM EDTA, 10 mM NaCl, 15 mM 2-mercaptoethanol, all adjusted to pH 7.4 at room temperature), poured into a column and eluted with 100 ml linear gradient from 10 mM to

750 mM in NaCl. Proteolytic activity was assayed in the presence and absence of Ca^{++} using 20 µl of the column fractions similar to the radioactive assay of Waxman (18) except that iodinated casein at 0.5 mg/ml was the substrate and the incubation was for 20 min at room temperature. Casein was iodinated according to the method of Moore, Williams and Lloyd (19). The assay for the endogenous inhibitor of the CANP was exactly identical to the proteolytic activity assay except that only 10 µl from the indicated fraction was added and every assay tube contained 10 µl from the pooled fractions having the highest CANP activity.

Aliquots of the fraction from the DEAE-cellulose column having the highest CANP activity were diluted with buffer A and added to liver membranes in a total volume of 36 µl which was 33 mM in HEPES, pH 7.4. The CANP-treated membranes were then phosphorylated at 0°C by making the sample 1 mM in Mn^{++} and adding [γ-^{32}P] ATP (6-12 µCi) for 2.5 min in the presence or absence of EGF in a total volume of 60 µl and analyzed by SDS-PAGE and autoradiography as described previously (16).

RESULTS: The autophosphorylating EGF receptor-kinase can be converted to a lower M_r form by an endogenous protease that is activated when A-431 cells are broken (10) in the presence of 1 mM calcium ion (11,12). Figure 1A shows that when A-431 cells were scraped from their culture flask into 1 mM Ca^{++}, the EGF-enhanced phosphorylation was decreased at M_r=180,000 (top arrow) and increased at M_r=160,000 (bottom arrow). Similar to A-431 cell membranes, liver membranes show EGF-enhanced phosphorylation of an M_r=180,000 protein which is presumably the EGF receptor-kinase (see Figure 1B, lanes 1,2). In contrast to A-431 cells, conversion of the autophosphorylating EGF receptor-kinase to a M_r=160,000 form did not occur when rat liver cells were broken in 1 mM Ca^{++} (see Figure 1B, lanes 3,4). Interestingly, CANP has been found in rat liver (18,20,21) along with an endogenous inhibitor which made detection of CANP in cytosols very difficult (18,21). Similar findings concerning the masking of CANP activity by its inhibitor have been made in other tissues (18,22). Since this endogenous CANP inhibitor may have prevented the calcium-activated proteolysis of the EGF receptor-kinase in the experiment with normal rat liver in Figure 1B, we used published procedures (18,22) to separate CANP from its inhibitor in rat liver cytosol. Cytosol proteins bound to a DEAE-cellulose column were eluted with a linear NaCl gradient. Inhibitor and CANP activity eluted at 200 mM and 400 mM salt, respectively. Since an aliquot of the fraction with the highest CANP activity was totally inhibited by an equal aliquot from either of two fractions with the highest inhibitor activity, there appeared to be more than enough inhibitor to totally inhibit CANP in rat liver cytosol.

Liver membranes were incubated with the DEAE-cellulose column fractions having the highest CANP activity in the absence and presence of Ca^{++} (Figure 1C and Figure 2,

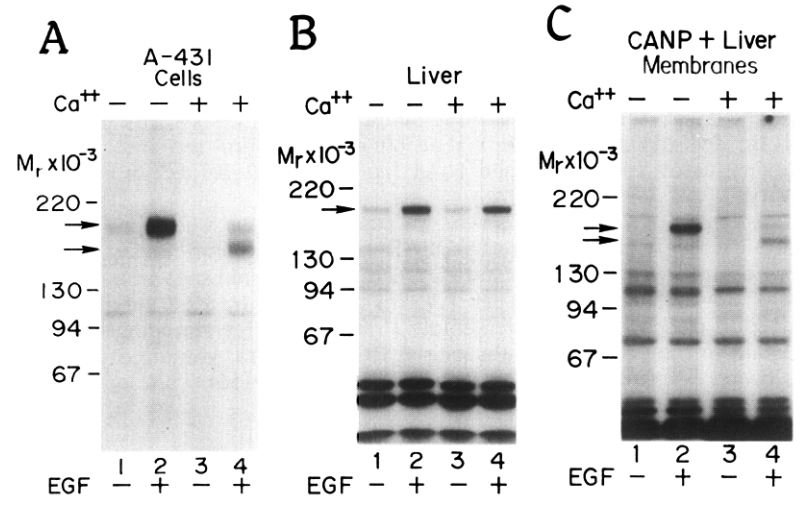

Figure 1: Effect of the presence of calcium during cell rupture on EGF receptor-kinase in membranes prepared from A-431 and rat liver cells.

A-431 (panel A) and rat liver (panel B) cells were disrupted in the absence (lanes 1,2) or presence (lanes 3,4) of 1 mM $CaCl_2$ and membranes were prepared as described in Materials and Methods. In panel A, 30 µl of crude A-431 membrane preparation was used for each lane and in panel B, 60 µg of liver membrane protein was used in each lane. In panel C, 55 µg of liver membrane protein prepared from liver cells disrupted in the absence of calcium were incubated for 5 min at room temperature in a total volume of 36 µl with 12 µl of a 1 to 2 dilution of the fraction from the DEAE-cellulose column having the highest CANP activity and in the absence (lanes 1,2) or presence (lanes 3,4) of 5 mM $CaCl_2$. This reaction was terminated by the addition of 6 nmoles of leupeptin and rapid cooling to 0°C. Samples in each lane were then incubated at 0°C with [γ-^{32}P]ATP in the absence (lanes 1,3) or presence (lanes 2,4) of 0.6 µg/ml EGF in a total volume of 60 µl. The samples were subjected to SDS-PAGE and an autoradiograph of the dried, stained gel is shown. The arrows indicate the positions of the 180,000 (panels A,B,C) and the 160,000 (panels A,C) M_r forms of the EGF receptor-kinase. The conditions for phosphorylation, electrophoresis and autoradiography have been described previously (16).

lanes 1-4). Phosphorylation with [γ-^{32}P] ATP and subsequent SDS-PAGE of either liver membranes alone or the membrane-protease mixture after incubation in the absence of Ca^{++} showed virtually identical EGF-enhanced phosphorylation of a single band of M_r=180,000. Incubating the liver membrane-protease mixture in the presence of calcium showed EGF-enhanced phosphorylation decreased at M_r=180,000 and increased at M_r=160,000 just as it was in A-431 cells lysed in the presence of calcium. Incubation of the liver membranes with calcium in the absence of added protease did not affect EGF-enhanced phosphorylation (data not shown). The above data show that, similar to the A-431 tumor cell line, the autophosphorylating EGF receptor-kinase in a normal tissue, rat liver, was also converted by calcium-activated proteolysis to a lower M_r form.

To further characterize the calcium-activated proteolysis of the autophosphorylating EGF receptor-kinase from rat liver, the effects of specific inhibitors of CANP on this proteolysis were studied. Figure 2 shows the effect of pre-mixing

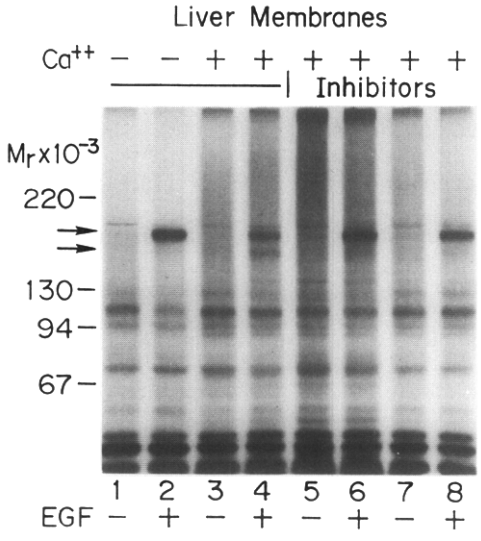

Figure 2: Effect of partially purified, active and inhibited, liver CANP on the EGF receptor-kinase in liver membranes.

In each lane, 50 µg of liver membrane protein were incubated with 12 µl of a 1 to 4 dilution of the fraction from the DEAE-cellulose column having the highest CANP activity in the absence (lanes 1,2) or presence (lanes 3-8) of 5 mM $CaCl_2$ for 5 min at room temperature in a total volume of 36 µl. In lanes 5,6 the CANP fraction was inhibited before the incubation by premixing with an equal volume of the supernatant of the heated (100°C, 5 min) fraction from the DEAE-cellulose column having the highest CANP inhibitor activity. In lanes 7,8 the added CANP was inhibited by premixing with 6 nmoles of leupeptin. Incubations were terminated and samples phosphorylated and analyzed as described in the legend for Figure 1 except EGF was present in lanes 2,4,6,8. An autoradiograph of the dried, stained gel is shown and the arrows indicate the position of the 180,000 and 160,000 M_r forms of the EGF receptor-kinase.

fractions from the DEAE-cellulose column, one with the highest CANP activity and one with the highest CANP inhibiting activity, and then incubating this mixture with liver membranes in the presence of calcium. Clearly the calcium-activated proteolysis of the EGF receptor-kinase was inhibited (See Figure 2, lanes 2,4,6). Since the specific CANP inhibitor does not lose its activity when heated (25), the supernatant from the inhibitor containing fraction after heating at 100°C for 5 min was used in all of these experiments. Leupeptin, a known inhibitor of CANP (18), also inhibited the proteolysis of the autophosphorylating EGF receptor-kinase by the partially purified CANP (compare lanes 2,4,8). Since both leupeptin and the partially purified specific inhibitor of CANP inhibited proteolysis of the EGF-receptor-kinase, this proteolysis is very probably due to CANP. As further proof, we note that bovine lung CANP purified to homogeneity converts, in the presence of Ca^{++}, the autophosphorylating EGF receptor-kinase from its M_r=180,000 to its M_r=160,000 form in both rat liver and A-431 membranes (data not shown).

DISCUSSION: When liver cells were lysed in the presence of calcium, the EGF receptor-kinase was not converted from the larger 180,000 M_r form to the smaller 160,000 M_r form, as had been observed with A-431 cells (11,12). Since the activity of CANP in liver is controlled by an endogenous protein inhibitor (18,25), CANP was separated from its cytosolic inhibitor and then incubated with liver membranes (Fig. 1). In this case the EGF receptor was converted to the smaller form indicating that the protease causing the conversion is nearly completely inhibited in normal liver cytosol. Recombining the separated inhibitor and protease blocked the conversion of the EGF receptor-kinase to the smaller form (Fig. 2). Significant levels of this inhibitor are presumably present in A-431 cells since proteolysis of the EGF receptor-kinase stopped 5 min after breaking these cells by scraping them from their culture flasks into 1 mM Ca^{++} (11,12) and no proteolysis was observed if calcium was not added until 5 min after scraping (11). Since CANP is a widely distributed protease (20) which degrades the EGF receptor in both A-431 and liver membranes, it may provide a highly regulated mechanism for modifying the EGF receptor in the cytoplasm of many cell types. In the intact cell, this modification by CANP may activate the EGF-enhanced kinase analogous to the irreversible activation of three other kinases (23,24,25) by CANP. Indeed the M_r=160,000 form of the receptor-kinase produced by CANP has been reported to be a better kinase for exogenous substrates than the M_r=180,000 form (1).

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