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Epidermal growth factor carrier protein binds to cells via a complex with released carrier protein nexin

(human fibroblasts/secreted binding mediator/covalent complex)

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ABSTRACT **Epidermal growth factor carrier protein (CP) is an arginine endopeptidase bound to epidermal growth factor (EGF) *in vivo* that processes pro-EGF to EGF and potentiates EGF action. Here, we provide a base for studying the biological functions of CP by showing that highly purified ^{125}I-labeled CP, free of contaminating EGF, is specifically bound and internalized by normal human fibroblasts in serum-free medium. The characteristics of the binding reaction, however, were unusual and not consistent with direct interaction of CP with cell surface receptors. Subsequent experiments showed that cellular binding of ^{125}I-labeled CP was mediated via a cell-secreted protein. We named the protein carrier protein nexin (CPN) because of its close functional similarity to protease nexin, which mediates cellular binding of thrombin or urokinase. Both CPN and protease nexin are secreted by cells, form covalent complexes with regulatory proteases in the extracellular environment, and mediate cellular binding of these proteases, apparently via a cell surface receptor for the nexin moiety of the complex. By several criteria, however, CPN and protease nexin are unique entities. This finding of a specific interaction of a growth factor carrier protein with cells suggests the possibility of additional physiological functions for these carriers in growth factor action or metabolism or both.**

Male mouse submaxillary gland epidermal growth factor (EGF) exists *in vivo* as a high molecular weight complex (M_r, ≈74,000) composed of two EGF molecules (M_r, 6,045) and two molecules of a specific carrier protein (M_r, ≈29,000) that possesses arginine endopeptidase activity (1). The EGF carrier protein (CP) proteolytically processes pro-EGF (M_r, ≈9,000) to the more commonly isolated EGF species (M_r, 6,045) by a cleavage approximately one-third the length of the pro-EGF molecule from its carboxy terminus (2). Importantly, CP remains tightly associated with EGF *in vivo* after proteolytic processing (2). In addition, it has been shown that EGF and CP spontaneously reassociate *in vitro* to form high molecular weight complexes (M_r, ≈74,000) that are indistinguishable from complexes isolated from submaxillary glands (1). It has also been reported that highly purified CP, free of contaminating EGF activity, potentiates the proliferative response of human foreskin fibroblasts (HF cells) to EGF (3).

To probe the mechanism of this potentiation by CP and to obtain information that might suggest additional functions, we examined the ability of CP to interact with cells. In initial experiments, we found that ^{125}I-labeled CP specifically bound to HF cells with a high affinity. The characteristics of the binding reaction, however, suggested a mechanism other than direct reversible interaction of CP with cell surface receptors. Closer examination showed that cellular binding of CP was mediated via a cell-secreted molecule in much the manner that cellular binding of thrombin or urokinase is mediated by cell-secreted protease nexin (4, 5). In this report, we describe the formation of covalent complexes between ^{125}I-labeled CP and the HF cell-secreted "protease nexin-like" molecules we have named CP nexin (CPN). These results show that nearly all of the ^{125}I-labeled CP specifically bound to cells was recovered in the form of ^{125}I-labeled CP–CPN complexes and that CPN mediates not only cellular binding but also the internalization of ^{125}I-labeled CP. Since protease nexin and CPN appear to be distinct, these results indicate the existence of a family of nexin molecules and suggest a general mechanism for the interaction of cells with regulatory proteases in the extracellular environment.

MATERIALS AND METHODS

Purification and Radioiodination of CP. CP was purified to apparent homogeneity from male mouse submaxillary glands essentially as described (2) with modifications introduced to ensure the absence of EGF in the final preparation. After isolation of pure high molecular weight EGF complexes, EGF and CP were dissociated in 0.05 M acetic acid and separated by column chromatography on Bio-Gel P-10. Void volume fractions containing CP were pooled, neutralized, and passed over an EGF affinity column prepared by coupling purified rabbit anti-EGF IgG to Sepharose CL-6B (6). EGF-free flow-through material was collected and pooled. Final purification of CP was accomplished on DE-52 cellulose as described (1). Aliquots of purified CP were stored at −20°C in phosphate-buffered saline (pH 7.2). Highly purified CP migrated as a single component in nonreduced $NaDodSO_4$/polyacrylamide gels (Fig. 1*A*, lane 1). In the presence of 50 mM dithiothreitol, CP dissociated into two closely migrating chains (M_r, ≈12,000 and 14,000) (Fig. 1*A*, lane 2). Purified CP was radioiodinated with Na^{125}I by using the immobilized iodogen method previously described (7). Specific activities were 35–70 Ci/g (1 Ci = 3.7×10^{10} becquerels). The radiopurity of iodinated preparations was evaluated by using 7% polyacrylamide native gel electrophoresis (8, 9). Radioiodinated CP migrated as a single homogeneous band coincident with the single Coomassie-stained band of CP on a parallel gel (Fig. 1*B*).

Binding Protocol. HF cells were cultured and prepared for experiments in 35-mm culture dishes as described (5). Cultures were incubated in serum-free medium for a minimum of 5 days before use in binding experiments. The binding medium was Dulbecco's modified Eagle's medium/20 mM Hepes, pH 7.4/

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Abbreviations: EGF, epidermal growth factor; CP, EGF carrier protein; CPN, CP nexin; HF cells, human foreskin fibroblasts; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Bistris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Chaps, 3-dimethyl(3-cholamido)propylammoniopropane-1-sulfonate; monotris, 2-hydroxyethylamino-tris(hydroxymethyl)methane.

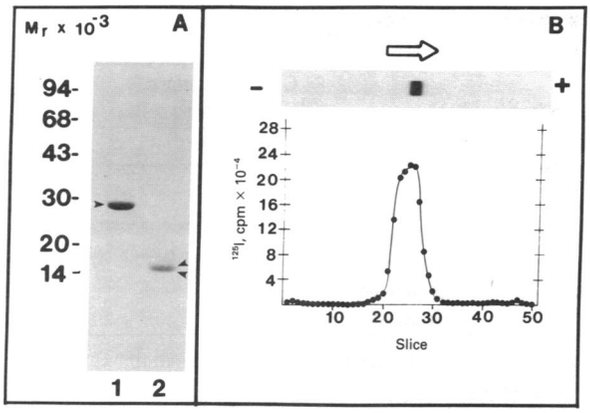

FIG. 1. Electrophoretic analysis of highly purified CP and ^{125}I-labeled CP. (*A*) Duplicate samples of highly purified CP were subjected to electrophoresis in the Laemmli system on 7.5–20% linear polyacrylamide gradient slab gels under reducing and nonreducing conditions. Lanes: 1, 5 μg of unreduced CP; 2, 10 μg of CP reduced with 50 mM dithiothreitol. (*B*) Highly purified CP (25 μg) and 10^6 cpm of ^{125}I-labeled CP were subjected to electrophoresis on parallel 7% native polyacrylamide gels in the Ornstein-Davis Tris glycine system (pH 9.5). The gel containing ^{125}I-labeled CP was frozen and sliced in 2-mm segments. (*Upper*) Coomassie-stained CP-containing gel (25 μg). (*Lower*) Plot of cpm vs. gel slice.

0.1% ovalbumin. Indicated concentrations of ^{125}I-labeled CP were added to cultures in 1 ml of binding medium. Binding reactions were terminated by aspirating the binding medium followed by six 2-ml rinses with phosphate-buffered saline (pH 7.2) at 4°C. The cells were then dissolved in boiling 1 M NaOH and assayed for radioactivity in a gamma counter at 85% efficiency.

To evaluate the role of released cellular components in the binding, serum-free HF cell-conditioned medium, freed of cells and debris, was harvested as described (4). Hepes (pH 7.0) was added to a final concentration of 20 mM. Ovalbumin was added to 0.1% unless otherwise noted. Indicated concentrations of labeled and unlabeled CP were added, and mixtures were incubated and then added to HF cell cultures for further incubation. Cell-bound radioactivity was analyzed as described above.

Samples of conditioned medium used to demonstrate ^{125}I-labeled CP–CPN complexes were by necessity prepared differently. Incubation with ^{125}I-labeled CP was carried out in the absence of ovalbumin in 5 ml of HF cell-conditioned medium. Then, phenylmethylsulfonyl fluoride was added to 2 mM, the reaction mixture was concentrated to 0.5 ml by pressure dialysis in an Amicon apparatus equipped with a PM30 membrane, 10 vol of electrophoresis sample buffer 0.025 M [*N*,*N*-bis(2-hydroxyethyl)glycine (Bicine)/0.01 M 2,2,-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bistris)] was added, and this mixture was concentrated by pressure dialysis to 0.5 ml. This process was repeated twice. Then, 10 vol of electrophoresis sample buffer/10 mM 3-dimethyl(3-cholamido)propylammoniopropane-1-sulfonate (Chaps, a zwitterionic detergent; ref. 8)/0.1% Na$DodSO_4$ was added, and the samples were concentrated to 0.5 ml. Glycerol and bromphenol blue were added to 10% and 0.01%, respectively. Aliquots of 0.05 ml were subjected to electrophoresis as described below.

Electrophoresis. To evaluate CP and ^{125}I-labeled CP, native gel electrophoresis (9, 10) and Na$DodSO_4$ gel electrophoresis using the Laemmli system were performed as described (11). For visualization of ^{125}I-labeled CP–CPN complexes by gel autoradiography, a discontinuous Na$DodSO_4$ gel system with a running pH of 8.2 was developed by using the equations of Jovin (12). Ten percent acrylamide gels or 7.5–15% total acrylamide linear gradient gels were cast as 15 × 8 × 0.1 cm slabs. Methylene-bisacrylamide was always 2% of total acrylamide. Separating gels were cast in 0.1 M 2-hydroxyethylaminotris(hydroxymethyl)methane (monotris) and 0.086 M HCl, pH 7.5. Potassium persulfate (200 μg/ml) and *N*,*N*,*N*′,*N*′-tetramethylenediamine (0.5 μl/ml) were added as polymerizing agents. In 10% acrylamide gels, the upper reservoir buffer was 0.043 M monotris/0.043 M Bicine/0.05% Na$DodSO_4$. The lower reservoir buffer was 0.1 M monotris (pH 7.6). The stacking gel was 3% total acrylamide in half-strength upper reservoir buffer without Na$DodSO_4$. Electrophoresis was toward the cathode at 7.5 mA constant current.

For molecular weight determinations, gradient gels were modified by using a 3% stacking gel containing 0.177 M Bistris/0.161 M HCl, pH 5.5. The upper reservoir buffer was 0.13 M Bicine/0.05 M Bistris/0.05% Na$DodSO_4$. The lower reservoir buffer was the same as for 10% acrylamide gels.

Gel Autoradiography. Gels were stained for 2 hr in methanol/acetic acid/H_2O (5:1:5)/0.05% Coomassie R-250 for 2 hr at 22°C. They were then destained in 5% ethanol/7% acetic acid. Slab gels were dried and prepared for autoradiography as described (5). Autoradiograms were exposed at −70°C for 4–24 hr.

Materials. All cell culture media and solutions were purchased from GIBCO. Cell culture plastics were from Falcon. Electrophoresis reagents were purchased from Bio-Rad with the exception of Bicine (Calbiochem) and Bistris (Aldrich). Monotris was synthesized as described (13) and recrystallized three times from absolute ethanol. Iodogen was purchased from Miles Laboratories; Na^{125}I was from Amersham. Autoradiographic supplies were purchased from Kodak. Chaps was synthesized and purified as described (8). Male mouse submaxillary glands were obtained from Pel-Freez. Chromatography resins were purchased from Bio-Rad and Pharmacia.

RESULTS

Binding of ^{125}I-Labeled CP to HF Cells. The time and temperature dependence of ^{125}I-labeled CP binding to HF cells was studied over a 5-hr time course. Binding was highly specific (>70%) and proceeded most rapidly at 37°C. Cellular binding did not, however, reach a steady state by 5 hr nor was "down regulation" observed (Fig. 2). In addition, there was rapid attainment of equilibrium at 4°C and a linear time course of binding at 22°C. These studies are in sharp contrast to the second-order binding reactions observed with most polypeptide ligands to their cell surface receptors. Overall, the binding kinetics of ^{125}I-labeled CP closely resembled the kinetics of a temperature-dependent enzymatic reaction.

Distribution of Bound ^{125}I-Labeled CP. To measure internalization of cell-bound ^{125}I-labeled CP, trypsin sensitivity was used to discriminate between surface-bound and internalized ligand (4). ^{125}I-Labeled CP was rapidly internalized at 37°C (Fig. 3). Its distribution reached an approximate steady state by 20 min, when 35% of total cell-bound ligand was internalized. Only a slight change in distribution was observed over the next 4 hr.

Effect of HF Cell-Conditioned Medium and Blockage of Cell Secretion on ^{125}I-Labeled CP Binding. The above similarities between ^{125}I-labeled CP cellular binding and the protease nexin-mediated binding of thrombin or urokinase prompted us to investigate the possibility that a cell-secreted mediator was also involved in ^{125}I-labeled CP binding. In the first of these experiments, the effect of HF cell-conditioned medium on binding was evaluated. ^{125}I-Labeled CP (300 ng/ml) was incu-

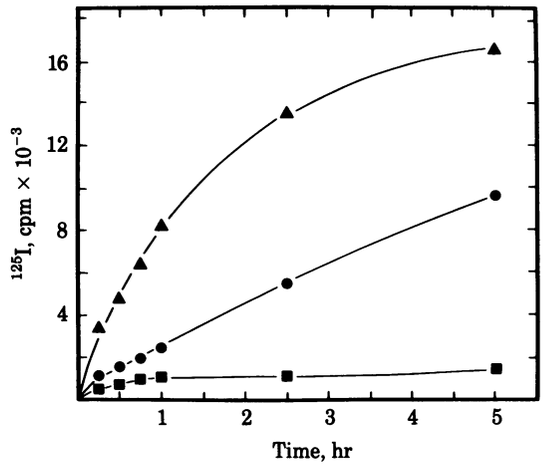

FIG. 2. Time and temperature dependence of ^{125}I-labeled CP binding to HF cells. Serum-free HF cell cultures (3×10^5 cells per plate) were incubated with ^{125}I-labeled CP (25 ng/ml; 80,000 cpm/ng) in binding medium at 4°C (■), 22°C (●), or 37°C (▲). At the indicated times, duplicate cultures were rinsed free of unbound ligand and assayed for cell-associated ^{125}I-labeled CP. Nonspecific binding (25% of total) determined in the presence of a 500-fold (mol/mol) excess of unlabeled CP was subtracted. Duplicates did not vary more than 10% from the mean.

bated for 30 min at 37°C with serum-free HF cell-conditioned medium and then added to HF cell cultures. Under these conditions, a 45% increase in cell-bound ^{125}I-labeled CP was observed over control cultures in which ^{125}I-labeled CP was incubated in fresh instead of conditioned medium (Table 1). Furthermore, the time of addition of a 500-fold molar excess of unlabeled CP made no difference when the binding assay was done in fresh binding medium. Whether added during the first incubation or at the time of addition of ^{125}I-labeled CP to cells, the amount of competition was the same (Table 1). In contrast, unlabeled CP was less effective in competition with cell-bound ^{125}I-labeled CP if added after the first incubation of ^{125}I-labeled CP with HF cell-conditioned medium (Table 1). These results suggest the formation of a stable complex between ^{125}I-labeled

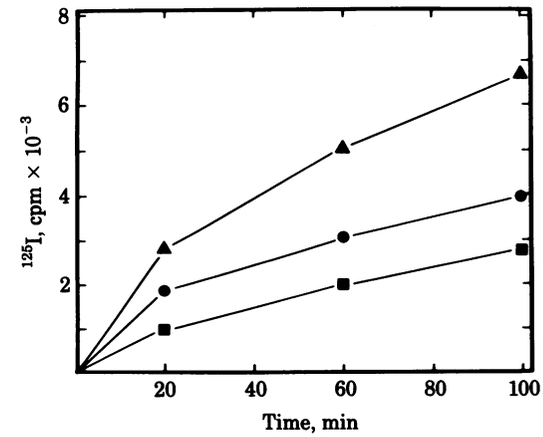

FIG. 3. Time course of internalization of cell-bound ^{125}I-labeled CP. Duplicate cultures of serum-free HF cells (3×10^5 cells per plate) were incubated with ^{125}I-labeled CP (25 ng/ml; 80,000 cpm/ng) in binding medium at 37°C. At the indicated times, ^{125}I-labeled CP was removed and the cultures were rapidly rinsed to remove unbound ligand. The cultures were then trypsinized for 10 min at 37°C to discriminate between trypsin sensitive (cell surface bound) and trypsin insensitive (internalized) as described (4). Nonspecific binding was subtracted as described in Fig. 2. ▲, Total bound radioactivity; ■, trypsin-sensitive radioactivity; ●, trypsin-insensitive radioactivity.

Table 1. Effect of previous incubation conditions on cellular binding of ^{125}I-labeled CP

First incubation		CP added before incubation with cells		Cell-bound ^{125}I-labeled
Medium	CP	^{125}I-Labeled	Unlabeled	CP, cpm
Fresh	^{125}I-Labeled	−	−	79,000
Fresh	^{125}I-Labeled	−	+	17,257
Fresh	Unlabeled	+	−	18,103
Conditioned	^{125}I-Labeled	−	−	114,000
Conditioned	^{125}I-Labeled	−	+	41,924
Conditioned	Unlabeled	+	−	20,397

Aliquots of HF cell-conditioned or fresh medium were incubated with ^{125}I-labeled CP (300 ng/ml) or unlabeled CP (10 μg/ml) at 37°C for 30 min. Then, ^{125}I-labeled CP (300 ng/ml) or unlabeled CP was added to some of the samples and these mixtures were incubated with HF cell cultures for 30 min at 37°C. Duplicates did not vary more than 3% from means.

CP and a component in HF cell-conditioned medium that is directly involved in cell binding. If the increase in cell-bound ^{125}I-labeled CP were simply due to the indirect effect of an "accelerator" of binding in conditioned medium, and not due to the formation of a stable complex as suggested, then the degree of competition of cell-bound ^{125}I-labeled CP should not have been affected by the time of addition of excess unlabeled CP. Since unlabeled CP did not compete effectively for the binding of *preformed* ^{125}I-labeled CP–CPN complexes to cells (Table 1), it appears that the complexes bind via the CPN portion.

To examine further the role of a cell-secreted component in ^{125}I-labeled CP binding, we conducted experiments under conditions in which its secretion was minimized during the binding assay. To do this, the binding was carried out at 4°C or at 37°C using cultures that had been treated with phenylarsine oxide. Both of these treatments strongly inhibit cell secretion and endocytosis (14). Binding was analyzed by using HF cell-conditioned medium previously incubated with ^{125}I-labeled CP at 37°C or conditioned medium to which ^{125}I-labeled CP was added without incubation prior to addition of cells. When binding to HF cells was done at 4°C, cultures that received ^{125}I-labeled CP previously incubated with conditioned medium at 37°C bound three times more ligand than cultures that received ^{125}I-labeled CP not previously incubated (Table 2). Nearly identical results were obtained in a parallel experiment in which cell secretion was inhibited by phenylarsine oxide at 37°C (Table 2). These results show the presence of a component in HF cell-conditioned medium directly involved ^{125}I-labeled CP cellular binding. In both experiments in which secretion was blocked,

Table 2. Effect of low temperature and phenylarsine oxide treatment on cellular binding of ^{125}I-labeled CP

Binding temperature, °C	Incubation with conditioned medium	Phenylarsine oxide treatment	Specific binding, cpm
4	+	−	6,750
4	−	−	2,250
37	+	+	5,750
37	−	+	1,980

In the first experiment, duplicate cultures of HF cells (3×10^5 cells per plate) were incubated for 30 min at 4°C with HF cell-conditioned medium containing ^{125}I-labeled CP (50 ng/ml) that had (+) or had not (−) been incubated for 30 min at 37°C prior to addition to the cells. In the second experiment, cultures were used that had been treated with 0.5 mM phenylarsine oxide for 1 hr at 4°C and then washed. Nonspecific binding (≈30% of total) has been subtracted. Duplicate cultures did not vary more than 10% from means.

significant specific binding was observed only when the component from HF cell-conditioned medium was supplied to the cells. These data also show that formation of ^{125}I-labeled CP complexes in conditioned medium is a temperature-dependent process that proceeds poorly, if at all, at 4°C. The temperature dependence of cell secretion and complex formation most likely explains the unusual temperature-dependent binding kinetics of ^{125}I-labeled CP and could also account for the low level of binding observed at 4°C (Fig. 1).

Identification and Partial Characterization of ^{125}I-Labeled CP–CPN Complexes. Previous studies showed that protease nexin released from cells forms covalent complexes with thrombin or urokinase and subsequently mediates cellular binding of these proteases via a cell surface receptor for the protease nexin moiety of the complex (4, 5). Because our data strongly indicated the role of a "protease nexin-like" component in ^{125}I-labeled CP cellular binding, it was important to determine whether we could identify the CP–CPN complexes. We initially conducted experiments using protocols identical to those used to visualize cell-bound ^{125}I-labeled thrombin–protease nexin complexes on NaDodSO$_4$/polyacrylamide gel autoradiograms. These attempts were unsuccessful and most likely reflected the lability of the CP–CPN complexes in the primary amine Tris buffer at the high pH of the Laemmli gel system. It was noted early in the protease nexin studies that exposure to primary amine buffers at high pH resulted in lower recovery of ^{125}I-labeled thrombin–protease nexin complexes on polyacrylamide gel autoradiograms (5). This was apparently the result of a primary amine-catalyzed breakage of an acyl link between thrombin and protease nexin that is important in stabilizing thrombin–protease nexin complexes. In the case of thrombin–protease nexin, this problem was circumvented by heating samples for electrophoresis in a tertiary amine Bistris buffer rather than the normally used Tris buffer. In the case of ^{125}I-labeled CP–CPN, however, complexes could not be detected as long as primary amine Tris buffers were used as electrolytes in the upper reservoir buffer. Thus, an electrophoresis gel system using a lower pH and secondary amines was developed to visualize ^{125}I-labeled CP–CPN complexes. This discontinuous NaDodSO$_4$ gel system was developed by using the equations of Jovin and used a secondary amine monotris buffer with a running pH of 8.2.

Cell-bound ^{125}I-labeled CP–CPN complexes were initially observed on straight 10% NaDodSO$_4$/monotris gels. HF cells were incubated with ^{125}I-labeled CP in the presence or absence of a large excess of unlabeled CP. Cultures were solubilized in 10 mM Chaps and prepared for electrophoresis as described in *Materials and Methods*. Combined autoradiograms of representative experiments are presented in Fig. 4. More than 90% of cell-bound ^{125}I-labeled CP was recovered in the form of two closely migrating complexes of $M_r \approx 125,000$ (Fig. 4*A*, lane 3). In addition, a 500-fold excess of unlabeled CP completely blocked the formation of these complexes (Fig. 4, lane 4). Thus, these complexes accounted for virtually all the specific binding of ^{125}I-labeled CP.

If CPN released by cells actually forms complexes with CP and mediates its binding to cells, as shown by the above binding studies, then it should be possible to show that ^{125}I-labeled CP–CPN complexes formed in conditioned medium. Indeed, when ^{125}I-labeled CP was incubated with serum-free HF cell-conditioned medium, a single major specific complex (M_r, $\approx$130,000) was formed between ^{125}I-labeled CP and a cell secreted component (Fig. 4*A*, lanes 1 and 2). In control lanes, only monomer (M_r, 29,000) and dimer (M_r, 58,000) forms of ^{125}I-labeled CP were observed (lanes 5 and 6).

While the gel system developed was excellent for the initial demonstration of ^{125}I-labeled CP–CPN complexes (Fig. 4), several modifications were next introduced to increase the resolution and gain a more accurate estimate of the M_rs of the complexes. These included the use of a Bistris stacking gel (pH 6.5), a higher NaDodSO$_4$ concentration in the solubilized buffer (1%), and the use of a 7.5–15% linear polyacrylamide gradient gel. ^{125}I-Labeled CP–CPN complexes were less stable under these conditions, as judged by their breakdown and the concomitant appearance of free ^{125}I-labeled CP (M_r, 29,000; Fig. 4*B*, lane 3; compare Fig. 4*A*, lane 3). Cell-bound ^{125}I-labeled CP–CPN complexes migrated as two major bands (M_r, $\approx$130,000 and $\approx$125,000) and a very minor band at $M_r \approx 120,000$ (Fig. 4*B*, lane 3). ^{125}I-Labeled CP–CPN complexes formed in conditioned medium migrated as one major band (M_r, $\approx$125,000) and two less prominent bands, one above and one below the major band. As before, the formation of ^{125}I-labeled CP–CPN complexes was strongly inhibited by the presence of a large excess of unlabeled CP during the binding reaction (Fig. 4*B*, lanes 2 and 4). Control lanes (5 and 6) showed the same pattern as in Fig. 4*A* (lanes 5 and 6), with only monomer and dimer forms of ^{125}I-labeled CP present.

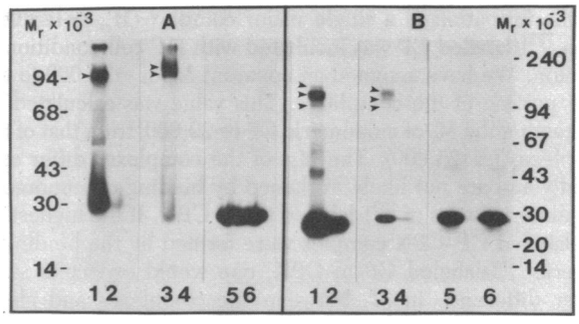

FIG. 4. Identification and characterization of ^{125}I-labeled CP–CPN complexes. (*A*) ^{125}I-Labeled CP (200 ng/ml, 50,000 cpm/ng) was incubated with HF cell-conditioned medium (10 ml) or HF cell cultures (2.5×10^5 cells per plate) for 30 min at 37°C in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 500-fold excess unlabeled CP. Cultures were rapidly rinsed, dissolved in 200 μl of solubilization buffer, and prepared for electrophoresis. Conditioned medium was concentrated to one-fourth volume and prepared for electrophoresis. Fifty microliter samples were subjected to electrophoresis on 10% polyacrylamide/NaDodSO$_4$ gels using a monotris/Bicine buffer system. Gels were prepared for autoradiography as described (5). Control lanes: 5, 6,000 cpm of ^{125}I-labeled CP not exposed to cells or conditioned medium; 6, 6,000 cpm of ^{125}I-labeled CP added to cells after solubilization. (*B*) Samples from an identical experiment were subjected to electrophoresis on 7.5–15% linear gradient polyacrylamide/NaDodSO$_4$ gels using a modified monotris/Bicine buffer system and a Bistris stacking gel to obtain accurate molecular weight estimates of ^{125}I-labeled CP–CPN complexes. Lane designations are as in *A*. ►, Marker proteins and ^{125}I-labeled CP complexes.

DISCUSSION

We have demonstrated specific binding and internalization of highly purified CP by cultured HF cells and have identified a cell-secreted component that mediates the binding process. This component, CPN, forms covalent complexes with ^{125}I-labeled CP in the culture medium and mediates the binding of ^{125}I-labeled CP to cells. It should be noted that CPN, like protease nexin, is secreted in large quantities by HF cells under serum-free conditions for periods of up to 3 weeks with frequent serum-free medium changes. Thus, it is not simply a serum component that is internalized by cells and subsequently released into the culture medium. Cell-bound ^{125}I-labeled CP was recovered in the form of high molecular weight complexes of $M_r \approx 130,000$, 125,000, and 120,000. The presence of a similar complex in HF cell-conditioned medium was verified by show-

ing the formation of a single major complex (M_r, ≈125,000) when ^{125}I-labeled CP was incubated with HF cell-conditioned medium. We have assigned an apparent M_r of ≈95,000 to the CPN portion of the complexes. This value was calculated by subtracting the M_r of monomeric CP (≈29,000) from that of the complexes (≈125,000). The M_rs of the complexes differ only slightly and are not likely explained by binding of monomeric and dimeric forms of ^{125}I-labeled CP to CPN. If the highest M_r ^{125}I-labeled CP–CPN complex were formed by the binding of dimeric ^{125}I-labeled CP to CPN, one would expect to see a larger difference in M_r between the complexes and also a greater proportion of dimeric ^{125}I-labeled CP in control lanes. However, only very small amounts of dimerized ^{125}I-labeled CP were observed on control lanes in autoradiograms (Fig. 4*B*, lanes 5 and 6). At present, we propose that ^{125}I-labeled CP binds to a single form of cell-secreted CPN, giving rise to predominately a single high molecular weight complex (M_r, ≈130,000; Fig. 4*B*, lane 3). This complex is subsequently bound to the cell surface via a receptor for the CPN portion of the complex. The appearance of two distinct and a very minor species of cell-bound ^{125}I-labeled CP–CPN complexes may arise from proteolysis by cellular proteases or the arginine endopeptidase activity of CP at the cell surface or during the process of internalization.

Several lines of evidence show that secreted CPN actually mediates binding of ^{125}I-labeled CP. First, cell-bound ^{125}I-labeled CP–CPN complexes were nearly identical in M_r to ^{125}I-labeled CP–CPN complexes formed in HF cell-conditioned medium and showed similar stabilities in the gel systems used. Second, prior incubation of ^{125}I-labeled CP with HF cell-conditioned medium resulted in a significant increase in cell-bound ^{125}I-labeled CP. Finally, blocking of cellular secretion by incubation at 4°C or by prior incubation with phenylarsine oxide at 37°C resulted in near total loss of cellular binding of ^{125}I-labeled CP. Partial restoration of binding activity was observed when cells were incubated with ^{125}I-labeled CP–CPN complexes previously formed by incubating ^{125}I-labeled CP with HF cell-conditioned medium at 37°C. Taken together, these results show that binding of ^{125}I-labeled CP is mediated by CPN in much of the same way that cell binding of thrombin or urokinase is mediated by cell-secreted protease nexin (4, 5).

CPN and protease nexin share many functional features, although they are probably different molecules. Both are cell secreted, form stable complexes with serine proteases in the extracellular environment, and mediate their binding to cells. Also, complexes formed by both are sensitive to exposure to primary amines at high pH (ref. 5; this report). Finally, complex formation between CPN and CP, as well as between protease nexin and thrombin or urokinase, appears to involve an acyl bond since derivatization of the catalytic site serine of CP (unpublished observation) or thrombin or urokinase (5) with diisopropyl fluorophosphate prevents complex formation with the appropriate nexin. Although CP appears not to form complexes with protease nexin, it is possible that certain proteases form complexes with both CPN and protease nexin. For several reasons, it is likely that protease nexin and CPN are unique entities. The first is size.

The apparent M_r of CPN in CP–CPN complexes is ≈95,000 while the apparent M_r of protease nexin in thrombin–protease nexin and urokinase–protease nexin complexes is ≈38,000 (1). Second, whereas the binding of ^{125}I-labeled thrombin–protease nexin complexes to HF cells is extremely heparin sensitive (4, 5), the binding of ^{125}I-labeled CP–CPN complexes is relatively unaffected by heparin (unpublished observations). Finally, thrombin–protease nexin complexes are much more stable to primary amines at high pH than are CP–CPN complexes. In fact, it was necessary to develop a primary amine-free gel system with a lower running pH to demonstrate the existence of ^{125}I-labeled CP–CPN complexes.

Protease nexin and CPN represent novel mechanisms for the binding of regulatory polypeptides to cells. In future studies, it will be important to examine the physiological significance of this process. Since the urokinase in urokinase–protease nexin complexes has been shown to be inactive, protease nexin and CPN might function as protease inhibitors and permit cells to monitor and control the levels of regulatory proteases in their environment. The fact that CP is a carrier protein for a polypeptide growth factor raises the possibility that CPN might play a role in EGF action by affecting its availability to cells.

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