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BINDING OF CESALIN, AN ANTITUMOR PROTEIN, TO CULTURED MAMMALIAN CELLS

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Summary

^{125}I-labelled cesalin binds to KB cells and plasma membranes in a specific and saturable manner. At 0°C the cesalin specifically bound to cells is completely displaceable by excess unlabelled cesalin, but at 37°C only 50% can be removed after incubation for 2 h. The extent of binding to plasma membranes has the following characteristics: it is increased following treatment of membranes with cholate; treatment with trypsin has no effect on binding and neither is the bound ^{125}I-labelled cesalin removed following digestion with trypsin; binding is not inhibited by several carbohydrates but is decreased to about one half by concanavalin A. In addition it is found that some degradation of cesalin occurs with KB cells, the specific binding to which is not enhanced by chloroquine, a lysosomotropic agent. No loss of binding in cells is seen after 4 h exposure to cesalin, suggesting no reduction in the receptors by internalization.

The data are consistent with a mechanism in which ^{125}I-labelled cesalin is rapidly bound at 37°C to a receptor on cell membranes through which the biological activity is effected. Slowly, some change in the bound cesalin occurs that prevents its complete displacement from the cells.

Cesalin, an antitumor protein isolated from the endosperm of *Caesalpinia gilliesii* [1], inhibits the biosynthesis of DNA, RNA and protein. The inhibition of nucleotide incorporation precedes that of amino acid by approx. 2 h

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Abbreviations: EGF, epidermal growth factor; LDL, low-density lipoprotein; hCG, human chorionogonado tropin; hPL, human placental lactogen.

and uridine incorporation is more rapidly inhibited than that for thymidine [2,3]. It does not, however, inhibit DNA synthesis in isolated nuclei [2].

Preliminary evidence has indicated that the effects of cesalin on tumor cells are brought about through specific binding to the plasma membrane. In spite of the intense study of membrane receptors for proteins, such as polypeptide hormones [4], toxins [5] and lectins [6], basic knowledge of the transmission of the effects of binding across the membrane is, in general, poorly understood. The present paper examines the binding of cesalin to KB cells and purified plasma membranes.

Experimental Procedure

Materials. Cesalin was prepared from the endosperm of *C. gilliesii* as previously described [2]. Cell culture media, serum, antibiotics, and trypsin solution (0.25% in Hank's buffered salt solution) were obtained from Grand Island Biological Company. $Na^{125}I$, carrier-free, was purchased from Amersham Corp. Hepes, bovine brain gangliosides, glucose oxidase (Type VII from *Aspergillus niger*) and bovine serum albumin were obtained from Sigma Chemical Co. Biobeads for enzymatic iodination were purchased from Bio-Rad Laboratories. Lactoperoxidase (from milk) was obtained from Boehringer Mannheim. Adenosine 5′-phosphate (disodium salt) was purchased from P-L Biochemicals, Inc. Elon (*p*-methylaminophenol sulfate), used as the reducing agent in the phosphate analysis, was obtained from Eastman Kodak Co. All other chemicals were reagent grade.

Cell line. KB cells (ATCC CCL 17) were obtained from the American Type Culture Collection, and maintained as monolayer cultures in Eagle's minimum essential medium with Earle's salts, supplemented with 10% (v/v) heat-inactivated fetal calf serum. The culture medium also contained penicillin and streptomycin at concentrations of 100 units/ml and 100 μg/ml, respectively.

^{125}I-labelled cesalin. Cesalin was iodinated either by using the method of Tower et al. [7] using lactoperoxidase and glucose oxidase, or by using the Enzymobead iodination reagent with immobilized enzymes from Bio-Rad. For the soluble-enzyme iodination, the following procedure was used: cesalin (25 μg) was dissolved in 25 μl of 0.1 M phosphate, pH 7.2, in a 1.5 ml Beckman microcentrifuge tube. To this solution were added 25 μl of lactoperoxidase (100 μl/ml in 0.4 M sodium acetate, pH 5.6), 20 μl of glucose oxidase (4 μg/ml of 0.1 M phosphate, pH 7.2), 1 mCi $Na^{125}I$ (10 μl of pH 7—11 NaOH solution), and finally, 20 μl of D-glucose (1%, w/v, in water). The reaction was allowed to proceed with occasional shaking for 30 min and stopped by the addition of 100 μl of NaN_3 (1 mg/ml in 0.1 M sodium phosphate, pH 7.2). The mixture was applied directly to a column (0.9 × 40 cm) of Sephadex G-100 at 4° C that had been equilibrated previously with 0.01 M Hepes buffer, pH 7.2, containing 0.1% bovine serum albumin and 0.15 M NaCl.

Iodination using Enzymobeads was carried out as follows: 25 μg of cesalin in 25 μl of 0.1 M phosphate buffer, pH 7.2 were added to 50 μl of Enzymobead suspension. To this suspension was added $Na^{125}I$ (1 mCi) and the reaction started by the addition of 50 μl of 1% (w/v) D-glucose in water. The reaction was stopped by centrifugation after 30 min and the supernatant applied to the

Sephadex G-100 column as above. The resulting ^{125}I-labelled cesalin from both procedures had a final activity of approx. 20 μCi/μg, with over 90% of the product being precipitable by 15% trichloroacetic acid.

Protein assay. Protein in the membrane fractions was determined by a Biuret assay according to the method of Goa [8], using bovine serum albumin as a standard.

5'-Nucleotidase assay. The plasma membrane marker, 5'-nucleotidase, was measured by the release of inorganic phosphate from the substrate, adenosine monophosphate. The sample (in 100 μl volume) was incubated at 37°C for 3—5 min with 0.8 ml of 0.01 M Tris buffer, pH 8.0, containing 0.1 M KCl, 0.01 M $MgCl_2$ and 0.01 M sodium potassium tartrate. The reaction was started by the addition of 100 μl of a 50 mM AMP solution in the same buffer. After 30 min at 37°C, the reaction was stopped by adding 1 ml of 10% (v/v) trichloroacetic acid. The tubes were centrifuged to pellet any precipitated material, and inorganic phosphate in the supernatant was measured by using a modification of the method of Fiske and Subbarow [9]: to 1 ml of the supernatant was added 1 ml of 1.25% (w/v) ammonium molybdate in 2.5 N H_2SO_4. After mixing, 1 ml of Elon reagent (15 g $NaHSO_3$ and 5 g of Elon in 500 ml aqueous solution) was added and mixed rapidly. After 20 min at 23—27°C, the absorbance at 660 nm was determined. Specific activity is expressed as μmol phosphate released per mg protein.

Iodination of viable KB cells. KB cells were washed, then removed from two T-150 Corning culture flasks (approx. $2 \cdot 10^7$ cells total) using 0.5 mM EDTA; the cells were centrifuged at $600 \times g$ for 5 min and resuspended by gentle pipetting in 0.5 ml of phosphate-buffered saline. Lactoperoxidase (50 μl), glucose oxidase (40 μl) and $Na^{125}I$ (2 mCi) were then added and the reaction was started with 40 μl of a solution of 1% (w/v) D-glucose, as described above. After 30 min at room temperature with occasional shaking, the cells were collected by centrifugation and washed three times with phosphate-buffered saline. More than 90% of the cells were able to exclude trypan blue, and contained a total of $6 \cdot 10^7$ cpm.

Preparation of KB plasma membranes. KB cells ($2 \cdot 10^8$), released by EDTA treatment, were resupended in a 10-vol. excess of 10 mM Hepes buffer, pH 7.2, containing 1 mM $MgCl_2$, (Hepes/Mg^{2+}) and swollen for 30 min at 0°C. All subsequent procedures were carried out at 0—4°C. The cells were homogenized by 20 strokes in a Wheaton Dounce homogenizer using a tight-fitting pestle A. The homogenate was centrifuged at $1085 \times g$ for 3 min. The resulting pellet (P1) was washed once with Hepes/Mg^{2+} buffer. The combined supernatants were centrifuged at $7500 \times g$ for 20 min, yielding a pellet (P2). The supernatant from P2 was finally centrifuged at $48\,000 \times g$ for 30 min, resulting in pellet 3 (P3) and the final supernatant (S3). Membrane fraction P2 was further purified by the two-polymer system of Brunette and Till [10]. The pellet P2, containing approx. 3 mg of protein, was suspended in 5 ml of the upper layer of the two-phase mixture. Lower layer (5 ml) was then added, and the solution centrifuged in a Beckman JA 7.5 rotor at 3000 rev./min for 15 min. The material at the interface was removed, and the procedure repeated twice more. The final interface material was washed with Hepes/Mg^{2+} buffer, and the pellet (P2/M) was subjected to centrifugation through a discontinuous

sucrose density gradient, consisting of 4 ml each of 45, 37, 33 and 29% sucrose (w/w) in Hepes/Mg^{2+} buffer. Following centrifugation at 50 000 × *g* for 16 h, the membrane fraction at the 33—37% interface was collected, washed and stored at −20° C in Hepes/Mg^{2+} buffer (P2/M-sucrose).

Binding assays. Assays were routinely carried out with $3—5 \cdot 10^5$ KB cells in 100 μl of phosphate-buffered saline incubated in 1.5 ml microcentrifuge tubes with 250 μl of bovine serum albumin/Hepes buffer and 20 000—50 000 cpm of iodinated cesalin in 50 μl bovine serum albumin/Hepes with or without the presence of excess unlabelled cesalin (50 μg). Following incubation at the appropriate temperature, the cells were pelleted in a Beckman microcentrifuge, washed once with bovine serum albumin/Hepes, and counted in a Beckman gamma counter, Model 300. Assays with membrane fractions were performed as above using the equivalent of 0.1—40 μg of protein in Hepes/Mg^{2+} buffer. Non-specific binding was defined as the amount of ^{125}I-labelled cesalin that could not be displaced by an excess of unlabelled cesalin (usually 50 μg).

Degradation of ^{125}I-labelled cesalin to acid-soluble material. Cells or membrane fractions were incubated with ^{125}I-labelled cesalin as described above. After incubation at the appropriate temperature, the cells were pelleted, washed once with bovine serum albumin/Hepes buffer, and counted. The cell-free binding medium was treated with trichloroacetic acid to a final concentration of 12.5% (w/v). After 3—5 min, the precipitate was collected by centrifugation and both the supernatant and precipitate were counted. The supernatant counts are expressed as a percentage of the total (precipitate and supernatant) counts.

Effect of trypsin on cell-bound ^{125}I-labelled cesalin. Cells ($3 \cdot 10^5$) were preincubated with ^{125}I-labelled cesalin for 60 min at 0, 24 and 37° C, as described above. At the end of the incubation, the cells were gently pelleted, and one-half was resuspended in bovine serum albumin/Hepes buffer without trypsin, while the other half was resuspended in buffer containing 0.1% trypsin. After incubation on ice for 30 min, the cells were pelleted and washed, and the counts in both pellets and supernatants measured.

Results

Purification of KB-plasma membranes and specific ^{125}I-labelled cesalin binding

The purification of plasma membranes from KB cells is shown in Table I. 5′-Nucleotidase was used as a specific plasma membrane marker to follow the fractionation. The specific activity of the 5′-nucleotidase in the sucrose gradient fraction (P2/M-sucrose) was 42 units/mg of protein, which was an increase of approx. 3-fold compared to the homogenate. Butters and Hughes [11,12] have reported on the purification of plasma membranes from KB cells, both in monolayer and suspension cultures. They found values of 59.6 units/mg of protein (monolayer cultures) and 61 units/mg of protein (suspension cultures) for membrane after purification in a sucrose gradient. The lower value of 42 units/mg of protein in the present study (Table I) may be due to a difference in the characteristics of the cells. This is supported by the fact that the cells used by Butters and Hughes showed an ($Na^+ + K^+$)-ATPase specific activity of 24.4 units/mg of protein, compared to the KB cells used in the

TABLE I

PURIFICATION OF PLASMA MEMBRANES FROM KB CELLS

Homogenates of KB cells were fractionated as described in Experimental Procedure. Specific activity of the plasma membrane marker, 5′-nucleotidase, is expressed as μmol phosphate liberated/h per mg protein. Specific ^{125}I-labelled cesalin binding is expressed as cpm bound per μg of protein. Protein was measured by the microbiuret method with bovine serum albumin as the standard.

Fraction	5′-Nucleotidase specific activity (units/mg protein)	Specific ^{125}I-labelled cesalin binding (cpm/μg protein)
Homogenate	16	278
P1	20	235
P2	25	556
P2/M	29	588
P2/M sucrose	42	1400
P3	24	351

present study with a value of about 5 units/mg of protein.

The specific binding of ^{125}I-labelled cesalin to the P2/M-sucrose membrane fraction was 5 times that to the homogenate. Fig. 1 shows the increase in specific ^{125}I-labelled cesalin binding with increasing plasma membrane purification. Both P2/M and P2/M-sucrose specifically bind 20—24% of the total counts added at protein amounts exceeding 10—15 μg, and maximum binding is reached in each fraction at 30—50 μg of protein.

Binding of ^{125}I-labelled cesalin to KB cells and purified plasma membranes

The specific binding of ^{125}I-labelled cesalin was characterized using both KB cells and purified membranes. With whole cells, the maximum amount of ^{125}I-labelled cesalin that can be specifically bound is 20—24% of the total

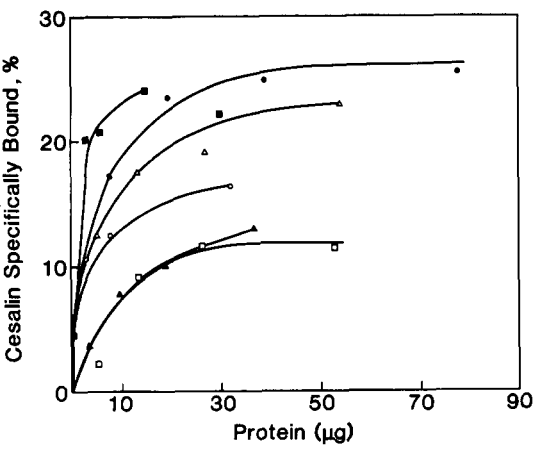

Fig. 1. Specific binding of cesalin to subcellular fractions of KB cells. Aliquots of the fractions of the purification of the purification of a plasma membrane from KB cells were assayed for specific ^{125}I-labelled cesalin binding by incubating 1—50 μg of membrane protein with 10 000—30 000 cpm of ^{125}I-labelled cesalin for 60 min at 25°C, as described in Experimental Procedure, with and without added excess (50 μg) unlabelled cesalin. All points are averages of duplicate or triplicate determinations. X———X, homogenate; □———□, P1 fraction; △———△, P2 fraction; ○———○, P3 fraction; ●———●, P2/M fraction; ■———■, P2/M sucrose fraction.

counts added, similar to that seen for purified membranes. The level of non-specific binding is usually less than 10% of the total counts added. In contrast, studies using ^{125}I-labelled insulin and ^{125}I-labelled diphtheria toxin have been complicated by the fact that the percent of label specifically bound was only 2—5% of the total counts added while the amount of non-specific binding was relatively high, 40—50% of the total counts [13,14]. Studies with epidermal growth factor (EGF) have shown remarkably low nonspecific binding, 1—6%, whereas the amount of ligand that binds specifically was as high as 60% [15].

Time course of ^{125}I-labelled cesalin binding

The fraction of the total counts of ^{125}I-labelled cesalin that were specifically bound increased with time but within 60 min at 0, 25, or 37°C, maximum binding to KB cells was attained (Fig. 2). Using $3 \cdot 10^5$ cells per assay, the level of binding at 0°C was 7% of the counts while 18 and 24% were bound at 25 and 37°C, respectively. Maximum binding with KB membranes required 90—120 min to reach equilibrium, slightly longer than for cells. Again, the

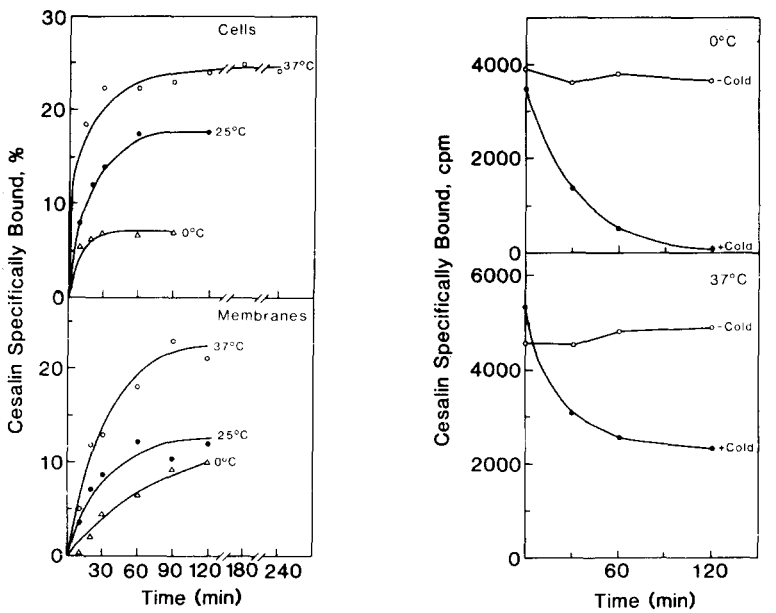

Fig. 2. Rate of specific binding of cesalin to KB cells and membranes. The increase in specific binding of ^{125}I-labelled cesalin to KB cells ($3 \cdot 10^5$) and membranes (7.5 μg) with time was measured over a period of 10 min to 4 h at 0, 25 and 37°C. At the end of the incubation time, cells or membranes were collected by centrifugation for 2 min in a Beckman microcentrifuge and the counts associated with the pellet were determined.

Fig. 3. Competitive displacement of pre-bound cesalin by excess unlabelled cesalin. KB cells were labelled for 60 min at 0 or 37°C by incubation of $3 \cdot 10^6$ cells with $3 \cdot 10^5$ cpm of ^{125}I-labelled cesalin in a final volume of 2.5 ml of bovine serum albumin/Hepes buffer. After 60 min, the cells were pelleted in a Beckman JS7.5 rotor at 3000 rev./min for 5 min. The cells were resuspended in 2.5 ml of bovine serum albumin/Hepes without (—cold) or with (+cold) 500 μg of unlabelled cesalin and incubated at 37°C. At the times indicated 250-μl aliquots (in duplicate) were removed and the counts associated with the cells were determined. A tube containing excess unlabelled cesalin throughout the binding and displacement steps was used as a measure of nonspecific binding, and these counts have been subtracted from each point.

binding at 0°C (10%) was significantly less than that at 37°C (24%) (Fig. 2).

It has been reported for both EGF [15,16] and insulin [17] that over the time course of association at 37°C, the amount of cell-bound ligand decreases. Insulin binding reaches a maximum at 10 min, and drops to approx. 60% of this level by 60 min. EGF binds maximally within 30—40 min and by 4 h only 15—20% of the initial maximal amount remains. This decrease in radioactive protein bound to the cells was apparently due to loss of functional receptors necessary for binding [16]. As seen in Fig. 2A the binding of ^{125}I-labelled cesalin remains at 24% during incubation at 37°C for 4 h. There appears to be no loss of receptor function or change in the ability of labelled cesalin in the medium to bind.

Dissociation of cell-bound ^{125}I-labelled cesalin

The binding of ^{125}I-labelled cesalin has apparently reached a steady state at 37°C after 60 min, and remained at this level for at least 4 h (Fig. 2A). This bound cesalin should then be displaceable by an excess of native cesalin if a true equilibrium has been established. Fig. 3 shows the results after preincubation of KB cells with ^{125}I-labelled cesalin, followed by addition of buffer or unlabelled cesalin to the washed cells. Very little dissociation up to 2 h was seen after resuspension of labelled cells in fresh buffer at 37°C, following either preincubation at 0 or 37°C. If excess native cesalin is added to washed cells that have been exposed to ^{125}I-labelled cesalin at 0°C, essentially all of the bound cesalin can be displaced from the surface. However, excess cesalin added to cells that were preincubated at 37°C displaced only 50% of the bound label after 2 h. Studies on the binding of EGF [15,16] and low-density lipoproteins (LDL) [18] have shown that both ligands completely dissociate from the cells at 37°C after resuspension in buffer over a time course of 1—3 h. Similar results are reported for diphtheria toxin binding to mammary gland membranes at 20°C [14].

It is seen that cesalin is still on the surface after binding to cells at 0°C, and although the association is tight such that dilution with buffer will not displace the label, excess unlabelled cesalin can compete for and completely displace the bound cesalin. After binding at 37°C, however, 50% of the bound cesalin is no longer available for competitive displacement, suggesting some irreversible interaction with a specific membrane component. The nature of the labelled cesalin removed by excess unlabelled protein was greater than 90% precipitable by trichloroacetic acid, suggesting that the bound protein remains intact at least up to 4 h after binding to the cell surface.

Cell-mediated degradation of ^{125}I-labelled cesalin

Several proteins have been shown to bind to cell-surface receptors, followed by internalization of the protein-receptor complex by a lysosomal-mediated process. Since it was found that some irreversible interaction of cesalin with KB cells was occurring at 37°C (see Fig. 3), the release of trichloroacetic acid-soluble material into the medium was compared at 0 and 37°C. Fig. 4 shows the appearance with time of acid-soluble fragments which continued to increase slightly after the extent of binding had reached a maximum. As expected, no degradation occurred at 0°C, which correlates with the complete removal of

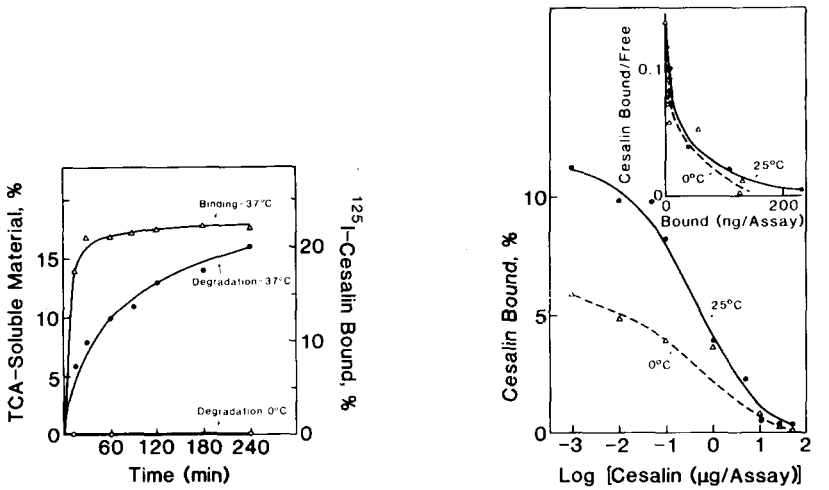

Fig. 4. The degradation of ^{125}I-labelled cesalin in the presence of KB cells. The increase in degradation of ^{125}I-labelled cesalin during the time course of binding to KB cells was measured as follows: $3 \cdot 10^5$ cells were incubated at 0 or 37°C with 10 000—30 000 cpm of ^{125}I-labelled cesalin in bovine serum albumin/Hepes as described in Experimental Procedure. At the times indicated, the cells were separated from the medium, washed and counted. The counts bound are shown in the figure as percent of the total counts added. Trichloroacetic acid was added to the medium to a final concentration of 12.5%. After 5 min, the trichloroacetic acid-pellet was collected by centrifugation in a Beckman microcentrifuge for 2 min. Both the trichloroacetic acid-pellet and the supernatant were counted, and the results expressed as the percent of the total counts in the binding medium which are trichloroacetic acid-soluble (TCA-soluble material).

Fig. 5. Scatchard analysis of cesalin binding to KB cells. Competition between ^{125}I-labelled cesalin and unlabelled cesalin for binding to KB cells was determined by the addition of increasing concentrations of unlabelled cesalin to the binding assays. KB cells ($3 \cdot 10^5$) were incubated at 25 and 0°C for 60 min with 10 000—15 000 cpm of ^{125}I-labelled cesalin in 250 μl bovine serum albumin/Hepes, containing 0.001—50 μg unlabelled cesalin per assay. The results are expressed as the percent bound vs. the log of the total cesalin concentration. These data were converted to total cesalin bound and bound/free to give a Scatchard plot (inset).

bound ^{125}I-labelled cesalin by excess cesalin at this temperature; iodinated cesalin incubated at 37°C with no cells was completely stable throughout a 4 h period. This cell-mediated degradation reaches 16% after 4 h at 37°C. When the cells were washed free of unbound ^{125}I-labelled cesalin after incubation for 60 min, no further release of radioactivity into the medium was observed (see Fig. 3).

To test the involvement of lysosomal-mediated degradation in the release of acid-soluble material during cesalin binding to KB cells, the effect of 100 μM chloroquine on this process was investigated. Whereas this concentration inhibited the degradation of LDL by 100% and EGF by 60%, little if any inhibition of the cesalin degradation was seen.

Effect of trypsin treatment on cell-bound cesalin

It has been reported that with increasing time of association, ^{125}I-labelled insulin bound to adipocytes became progressively more resistant to removal by 0.1% solution of trypsin [21]. After preincubation with ^{125}I-labelled cesalin for 60 min at 0°C, 13.1% of the bound radioactivity was removed by trypsin,

where at 25 and 37°C, 10.6 and 8.9%, respectively, cell-bound vesalin was released.

Effect of trypsin on plasma membrane-associated receptor activity

Earlier data from this laboratory showed that the use of EDTA or trypsin to remove cells from the flask surface had no effect on binding of ^{125}I-labelled cesalin [2]. The effect of trypsin on KB-plasma membrane receptor activity was examined further, and compared to the amount of trypsin-sensitive surface protein. Isolated KB membranes (50 μl) were incubated with 0.25% trypsin at 25°C for 60 min. The membranes were pelleted, resuspended in the original volume of bovine serum albumin/Hepes buffer, and the binding of ^{125}I-labelled cesalin was measured. No decrease in binding was found, 5000 cpm being specifically bound with treated and untreated membranes. Using KB membranes, which had been prepared from the enzymatic iodination of KB cells, it was found that greater than 80% of this label could be removed by trypsin. These results suggest that the cesalin receptor is not protein, or is resistant to trypsin, or is 'buried' within the membrane and not accessible to trypsin cleavage. Similarly, it was found that treatment of membranes with sodium cholate (0.5%, w/v) did not decrease the ability of cesalin to bind, and in fact, a reproducible stimulation of greater than 25% in the specific binding was observed. This result suggests not only that the receptor might be an integral membrane conponent, but that stripping away of external proteins by cholate solubilization might unmask more sites.

Displacement of cell-bound ^{125}I-labelled cesalin by unlabelled cesalin

The results presented above strongly suggest that the cesalin-receptor interaction is not a simple bimolecular reversible reaction. Once bound, the cesalin can only be completely displaced by high concentrations of cesalin and only at 0°C where degradative processes are minimized. The ability of increasing amounts of unlabelled cesalin (0.001—100 μg per assay) to displace ^{125}I-labelled cesalin is shown in Fig. 5, for binding to KB cells at 0 and 25°C. Conversion of these displacement data by Scatchard analysis results in curvilinear plots at both temperatures. Although other workers have explained this type of plot by assuming the existence of high- and low-affinity binding sites, it would be difficult to calculate any meaningful affinity constants in the case of cesalin where equilibrium apparently does not exist either at 0 or 25°C (see Fig. 3).

Discussion

Cesalin, like many biologically active polypeptides, exerts its biological effect by first interacting with a specific membrane receptor. This binding capacity copurified in the fractionation of cell homogenates with a known plasma membrane marker, 5′-nucleotidase, which has been demonstrated also for prolactin [23], diphtheria toxin [14], insulin [17], glucagon [24] and growth hormone [25].

Quantitative analysis of protein-receptor interactions is complex. Classical analyses using the Scatchard model [26] are dependent on several requirements which are often not met: (i) the protein ligand is homogeneous; (ii) the labelled

and unlabelled proteins behave identically; (iii) the protein-receptor interaction behaves as a simple bimolecular reversible reaction; and (iv) equilibrium of binding can be achieved. If these criteria are met, quantitative analysis will yield linear Scatchard plots, such as has been shown for ovine prolactin [23, 27] and human growth hormone [25], and a close approximation of K_a or K_d values can be obtained. However, many protein-receptor interactions have resulted in nonlinear plots, the most notable being insulin where progressive saturation of receptors reduces their affinity for the hormone, so-called negative cooperativity [28]. This type of site-site interaction is also seen in analyses of dissociation data, since the dissociation rate will increase as unlabelled hormone is added, thereby causing a reduced affinity for the bound ^{125}I-labelled insulin.

It is evident from the data in the present study that the cesalin-receptor process is not a simple bimolecular reversible reaction. Once bound to its surface receptor, the labelled-cesalin remains associated with the membrane for several hours after excess protein has been removed. Addition of unlabelled cesalin can remove the bound protein, but only below certain temperatures. At 37° C, an irreversible process has occurred such that only 50% of the bound cesalin can be displaced. This alteration is apparently occurring slowly or not at all at 0° C, at which temperature essentially all of the bound protein can be dissociated.

The relationship between degradation of a biologically functional protein and its binding to a specific receptor necessary for carrying out that function is often difficult to determine. EGF [15,16,19], LDL [18,20] and human chorionogonado tropin (hCG) [29] have been shown to bind to cell surface receptors, followed by internalization of the receptor-protein complex through endocytosis and degradation in the lysosomes. It has not been shown for EGF whether degradation is required to elicit a biological response. Likewise, no correlation of hCG internalization and activation of steroidogenesis were found. On the other hand, it has been proposed that endocytosis of LDL is an important transport mechanism to distribute cholesterol to the cells and to regulate cholesterol synthesis by feedback inhibition.

In hormone-receptor interactions, separate degradative and binding sites have been demonstrated. Human placental lactogen (hPL) has two distinct interaction sites on mammary cells, one responsible for the biological activity, and one involved in degradation of the bound hormone [30]. That these were independent sites was demonstrated by the fact that the receptor sites could be solubilized using Triton X-100, whereas sites for active degradation remained in the particulate fraction. Also, prolactin could block hPL interaction with receptor sites, but not the degradative sites. Separate binding and degradation sites on liver plasma membranes are similarly seen for glucagon [31] and insulin [17]. In none of these instances, however, has a direct dependence of hormone action on the degradative process been found.

Cesalin appears to undergo some alteration after binding to the plasma membrane at 37° C and an increase in acid-soluble counts is seen in free solution. Both EGF [15,16] and LDL [18] showed considerably higher levels of acid-soluble material than was seen with cesalin. Within 3 h at 37° C, 65% of the labelled LDL has been converted to low molecular weight fragments. Similarly,

after 2 h at 37°C, 85—95% of added ^{125}I-labelled EGF has been released as acid-soluble material. The process whereby EGF [19] and LDL [20] are transported into the cell following interaction with cell surfaces may be mediated by the lysosomes. This was supported by the fact that two specific lysosomotropic agents, NH_4^+ and chloroquine, interfered with the release of low molecular weight material from these two protein ligands. Little if any inhibition of the degradation of cesalin was seen in the presence of 100 μM chloroquine, which was sufficient to inhibit completely the degradation of LDL and to reduce the degradation of EGF by 60%. Preliminary chromatographic evidence indicates that some of the labelled cesalin is partially degraded at 37°C and stays bound to the membrane. The small cleaved fragments may thus account for the acid-soluble counts released into the binding medium (Fig. 4). The resulting bound and degraded cesalin may be neither readily displaced by excess native cesalin (Fig. 3) nor transported into the cell. However, the degradation at 37°C may be due also to a nonspecific proteolysis of the free ^{125}I-labelled cesalin mediated by the KB cells. It is not known which of these two processes is predominant.

Many hormone receptors are sensitive to trypsin treatment. For example, insulin becomes increasingly resistant to trypsin cleavage as the time of association increases [21]. After 2 min, 75% of the bound insulin was released, while after 30 min, 40% of the total could be removed. Similar experiments with EGF bound to human fibroblasts showed that after incubation with ^{125}I-labelled EGF at 0°C, 47% of the label could be removed with 0.25% trypsin, whereas only 10% was released from cells incubated at 37°C [22]. In comparison, both the cesalin receptor and the receptor-cesalin complex are resistant to change by trypsin. No effect on receptor activity was noted after treatment of the cells with 0.25% trypsin. A small percentage (13.1%) of bound cesalin at 0°C can be released with trypsin and this percentage decreases to 10.6% at 25°C and 8.9% at 37°C, which further suggests that cesalin may undergo an alteration after binding at these higher temperatures. Cholate treatment of cell membranes, on the other hand, actually stimulates receptor activity, and may be due to a solubilization of sterically interfering proteins. Conversely, treatment of the cell membrane with 2 μM concanavalin A inhibits the binding of cesalin by 46%. Although concanavalin A has been shown to interfere in the binding and degradation of LDL in human fibroblasts [20], it is not certain at the present time whether it shows a specific inhibition of cesalin binding, or is simply binding to a concanavalin A-specific receptor on the KB cell and masking the cesalin receptor. It was noted that macromomycin [34] does not compete with cesalin in its binding to the membrane.

Polypeptide antitumor agents have not been well studied with regard to their mechanism of interaction with cell-surface receptors. Although the structure and mode of action have been described in detail for neocarzinostatin [32,33] and, to a lesser extent, for macromomycin [34,35], cesalin is the only antitumor protein which has been shown to react specifically with a membrane receptor. Since the sensitivity of tumor cells to cesalin is proportional to the degree of specific binding to the plasma membrane, this membrane interaction becomes the first chemotherapeutic event in the recognition of the tumor cell for which cesalin is cytotoxic. The emphasis of future research with the anti-

tumor proteins will be directed towards an understanding of the nature of the binding and the specificity of this event. If a receptor-mediated binding is obligatory then a highly specific transport or transmembrane signalling is expected, together with equivalent specificity for cell type. The tumor-specific membrane proteins may present the source of this cell specificity and target some tumor cells for selective sensitivity to the cytotoxic antitumor proteins.

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