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CARCINOEMBRYONIC ANTIGEN BINDING TO KUPFFER CELLS IS VIA A PEPTIDE LOCATED AT THE JUNCTION OF THE N-TERMINAL AND FIRST LOOP DOMAINS

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A 11kD glycopeptide has been isolated by pepsin digestion of carcinoembryonic antigen (CEA) that is rapidly endocytosed by isolated rat Kupffer cells and lung alveolar macrophages. Uptake of this glycopeptide by the isolated cells can be inhibited by excess unmodified CEA. Removal of the N-linked oligosaccharide chains by N-glycanase did not alter cellular uptake but reduced the MW to approximately 5500. A seventeen amino acid N-terminal sequence locates this peptide at the junction of the N-terminal and first loop domain of CEA. It is suggested that the recognition of a peptide sequence in this area of CEA is responsible for its clearance from the circulation.

Carcinoembryonic antigen (CEA) is a highly glycosylated protein whose measurement in serum has been used as an indicator of prognosis, tumor recurrence and for monitoring therapy in patients with colorectal and other carcinomas (1). CEA is composed of a single polypeptide chain of 668 amino acids, with 28 potential sites for N-linked glycosylation (2). Complex carbohydrate chains largely of the tetra-anntenary type comprise over 50% of the molecule (3). CEA is one of a large family of molecules that include non-specific cross reacting antigen (NCA), the biliary glycoproteins (BGP_{a-d}) and the pregnancy-specific β_1-glycoproteins (4). These family members have a high level of sequence homology and belong to the much larger immunoglobulin supergene family (5). A domain model for CEA has been suggested (6,7) and comprises of a 108 residue N-terminus which is relatively free of carbohydrate, three 178 residue repeating loop domains each with two disulfide bridges and a 26 residue hydrophobic C-terminus that may be lost when the molecule is secreted (8,9).

CEA is removed from the circulation by the liver (10). Specific binding proteins for CEA and NCA have been demonstrated on both rodent and human Kupffer

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cells (11,12). Previous studies with isolated rat Kupffer cells showed that the area of CEA that binds to these proteins is peptide in nature (11). A more precise definition of the binding site is required and the evidence presented in this study localizes this binding site to the N-terminal end of the first disulfide loop domain.

MATERIALS AND METHODS

CEA Purification

Human carcinoembryonic antigen (CEA) was obtained from hepatic metastases of colorectal cancers by perchloric acid extraction followed by chromatography on Sepharose 4B and Sephadex G200. The purity of the preparations was determined by SDS-PAGE, HPLC analysis and by immunoreactivity as previously described (11). The CEA was homogeneous by the above criteria and appeared as a single band of 180-200 kD on Western blot analysis using a polyclonal antiserum raised in goats (a gift from Dr. D. Darcy Institute of Cancer Research, London) that recognizes both CEA and non specific cross reacting antigen (NCA).

Pepsin Digests

CEA (2mg) was incubated with pepsin (0.04mg) (Sigma Chemical Co.) in 0.1 M-acetate buffer, pH 2.2 at 37°C for 16 hours. An inital separation of the digest was carried out by centrifuging through a Centricon-30 membrane (Amicon). The high and low molecular mass fractions were each fractionated by chromatography on an Ultropac TSK G3000SW column. Individual peaks from the chromatography were examined by SDS-polyacrylamide-gel electrophoresis.

Isolated glycopeptides were treated with N-glycanase (a gift from Dr. J. Elting, Molecular Diagnostics, CT) to remove N-linked oligosaccharide chains. The ^{125}I-labeled peptide was boiled for 5 minutes in 0.05% SDS/10% DTT. The denatured glycopeptide was incubated at 37°C in 0.15M phosphate buffer pH 8.5 with 0.01M phenanthroline and 1.5% NP40 and the N-glycanase added (~50 units) in 3 aliquots over 16 hours. The reaction mixture was centrifuged through a Centricon 3 membrane and examined by SDS-PAGE.

Isolation of Rat Kupffer Cells and Lung Alveolar Macrophages

Kupffer cells were harvested from the livers of anesthetized male Sprague-Dawley rats (250-300 g) by collagenase perfusion, differential centrifugation and final purification on a metrizamide gradient (17.5 %) as previously described (11). Greater than ninety-five percent of the resulting sinusoidal cells were viable by trypan blue dye exclusion. Sixty-five percent of the cells were identified as macrophages by nonspecific esterase and peroxidase staining and sixty-four percent of the cells phagocytosed colloidal carbon and 0.8 um latex beads. The preparations contained less than 1% hepatocytes, as counted microscopically. Alveolar macrophages were obtained by saline lavage of the lungs following perfusion of the liver and examined for purity as described for Kupffer cells. Lung lavage cells were >90% macrophages and >95% viable (13).

Uptake of Peptides by Isolated Lung and Liver Macrophages

Peptides isolated from the pepsin digests were labeled with ^{125}I using the Chloramine T reaction (14). Labeling was approximately 5 mCi/mg of peptide in each case. Suspensions of Kupffer cells or alveolar macrophages were incubated at 37°C with the labelled peptide in buffered RPMI 1640 containing 1% bovine serum albumin. For inhibition of uptake experiments unmodified CEA or NCA was

included in the reaction mixtures. The incubation mixtures were sampled at various times, and the cells separated by centrifugation through an oil phase at 11,000 rpm for 5 minutes using an Eppendorf microcentrifuge. The oil phase used for Kupffer cells was dibutylphthalate:dioctylphthalate (3:1), while for alveolar cells it was silicone oil:mineral oil (4:1) (11,13). Both glycopeptides and N-glycanase deglycosylated glycopeptides were examined in this assay.

Peptide Sequencing

Peptides were sequenced following electrophoresis on 15% polyacrylamide gels and electrophoretic transfer to PVDF membranes (Immobilon, Milipore, Bedford, MA). Gas-phase sequencing was carried out on the Coomassie blue stained bands from the PVDF membranes (15).

RESULTS AND DISCUSSION

Our inital attempts to characterize the region of the CEA molecule that binds to the Kupffer cell suggested that it was peptide in nature (11,16). The present study confirms the peptide nature of the interaction and locates the binding site to an area of approximately 50 amino acids at the beginning of the first disulfide loop domain of CEA.

Pepsin digests of CEA produce a limited number of small glycopeptides (17). The three major species were 11, 12.5 and 16kD respectively as well as a large fragment of approximately 120kD. From this mixture the 11kD glycopeptide was

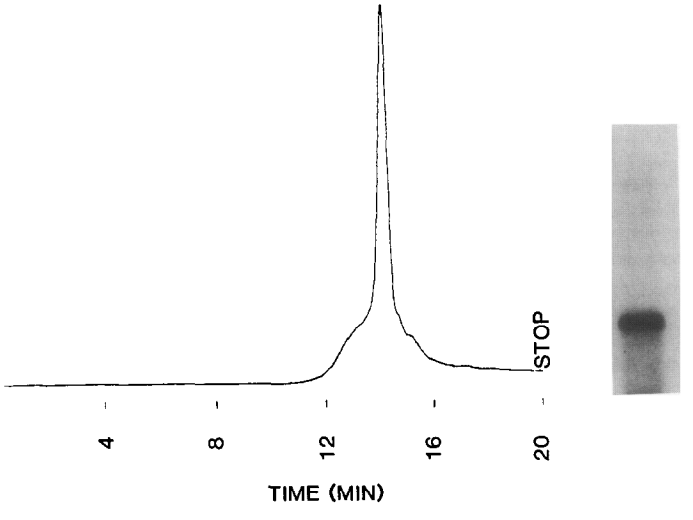

Figure 1.
H.p.l.c. profile of the 11kD peptide run on an Ultropac G3000SW column. The major peak (retention time 14.5min) was collected and run on 15% polyacrylamide gels prior to transfer to PVDF membranes for sequencing. The inset shows the autoradiogram of the ^{125}I labeled glycopeptide run on a 15% SDS-polyacrylamide gel.

isolated by HPLC and SDS-PAGE (Fig. 1). This glycopeptide bound to both rat liver Kupffer cells and lung alveolar macrophages. Other glycopeptides isolated from the digest including the large fragment were not capable of specific binding to the isolated cells. Binding of the 11kD glycopeptide to both Kupffer cells and alveolar macrophages was inhibited by incubation with excess CEA (Fig. 2). Treatment of the glycopeptide with N-glycanase resulted in a decrease in its MW to approximately 5,500 while its ability to bind to isolated Kupffer cells was retained. This MW change suggests that the glycopeptide contains two complex N-linked oligosaccharide chains of the type described by Chandrasakeran et al (3) and these oligosaccharides are not involved in Kupffer cell binding. This represents the first definitive proof of the peptide nature of the binding site. Previous studies though suggestive of peptide binding used CEA modified by multiple Smith degradations and still retained some carbohydrate (11,16).

Gas phase sequencing of the 11kD glycopeptide from the PVDF membranes resulted in a 17 amino acid N-terminal sequence (Fig. 3) that matched the known CEA sequence (2) between amino acids 105 and 122. This places the binding area of CEA near the beginning of the first loop domain. With the exception of residue 105 where Arg is replaced by His and residue 121 where Lys is replaced by Asn an identical sequence is found in NCA (18). NCA also binds to Kupffer cells via the same site as CEA but with a lower affinity (11).

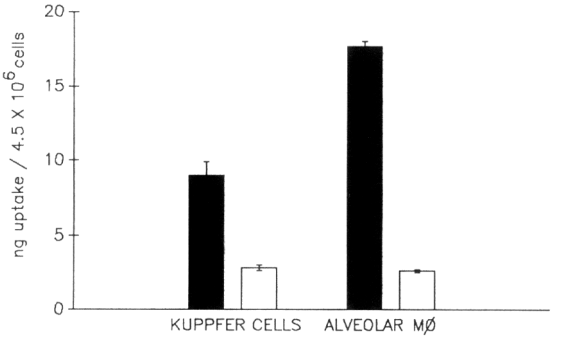

Figure 2.
In vitro uptake of radiolabeled peptide (2.8 μg/ml) by isolated rat alveolar macrophages and Kupffer cells (4.5×10^6) after 45 minutes at 37°C is represented by the solid bars, inhibition of uptake by a 250 fold molar excess of unlabeled CEA is represented by the open bars.

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Peptide:
    X--Val-Tyr-Pro-Glu-Leu-Pro-Lys-Pro-Ser-Ile-Ser-Ser--X--Asn--X--Lys-Pro-

CEA:
   Arg-Val-Tyr-Pro-Glu-Leu-Pro-Lys-Pro-Ser-Ile-Ser-Ser-Asn-Asn-Ser-Lys-Pro-

NCA:
   His-Val-Tyr-Pro-Glu-Leu-Pro-Lys-Pro-Ser-Ile-Ser-Ser-Asn-Asn-Ser-Asn-Pro-


-X-, - Amino acid not found
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Figure 3.
N-terminal sequence obtained for the 11kD glycopeptide. Comparison with the corresponding known sequences of CEA and NCA.

The identification of this binding sequence located to the beginning of the first loop domain now allows us to look even more closely at this peptide binding site. Use of synthetic peptides should narrow down the sequence required for binding even further. It has been suggested that the binding of CEA to Kupffer cells may be involved in the development of hepatic metastases from colorectal cancers by allowing the tumors to adhere to these cells in the sinusoid (19). The suggestion has also been made that CEA functions as a homotypic adherence molecule in tumors and in fetal colon (20). This idea is based on interactions between mouse L cells transfected with the CEA gene, cells expressing CEA form aggregates which can be inhibited by anti-CEA antibodies. Similar experiments have also been carried out using transfected CHO cells demonstrating both homophilic interaction between CEA molecules and heterophilic interactions between CEA and NCA (21). Thus, CEA bound to Kupffer cells may be able to interact with CEA on tumor cell surfaces in a similar homotypic fashion (22). Peptides based on the Kupffer cell binding peptide described here may possibly act as inhibitors of these interactions and influence the development of hepatic metastases.

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