Receptor-linked Proteolysis of Membrane-bound Glucagon Yields a Membrane-associated Hormone Fragment

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The consequences of ligand association with hepatic glucagon receptors are complex in that (a) high and low affinity binding sites each contribute substantially to glucagon association with isolated hepatocytes (1, 2) and hepatic plasma membranes (3–6), (b) glucagon at appropriate concentrations has the ability both to stimulate and to inhibit hepatic adenyl cyclase (7–9), (c) glucagon actions on hepatocyte metabolism can apparently arise from separate signal-transducing systems involving the generation of either cyclic AMP or inositol triphosphate (10), and (d) a variety of glucagon-related peptides. Overall, our results identify a proteolytic modification of glucagon that is linked to the binding of ligand to high affinity GTP-dependent receptors and the existence of a physically distinct state of receptor in which the binding site is tightly filled by a ligand fragment.

EXPERIMENTAL PROCEDURES

Materials—Glucagon was obtained from Elanco (Indianapolis, IN); Na2SO4 was from Amersham Corp. DFDNB and BS3 were from Pierce Chemical Co., and acrylamide, SDS, TEMED, sodium persulfate, bisacrylamide, molecular weight standards, and Bio-Gel P2 were from Bio-Rad. All other chemicals were reagent or higher grade and were obtained from standard sources.

Purification of Canine Hepatic Plasma Membranes—Membranes were prepared by a modification of the method of Neville (20). Finely minced canine liver (500 g) was homogenized using a Polytron tissue homogenizer at a setting of 3–4/10 for 5 min in aliquots of 0.5 ml of buffer containing 0.5 mM CaCl2, 1 mM NaHCO3, pH 7. The homogenates were filtered through four layers of cheesecloth, and the particulate material was washed by pelleting (20,000 × g for 30 min) and resuspending in the same buffer three times by use of 6, 6, and 2 liters of buffer, respectively. The final pellets were suspended in 85% (w/v) sucrose to make a suspension containing 63% sucrose (total volume about 150 ml) which was divided into 7-ml aliquots in 50-ml polycarbonate centrifuge tubes. The plasma membranes were separated from other material by flotation through step gradients consisting of 10 ml each of 59, 54, and 33% sucrose. Subsequent to centrifugation in a Sorvall model RC-3B centrifuge (5000 × g for 18 h), membranes appearing at the 54%-33% sucrose interface were removed by use of a Pasteur pipette; the membranes were diluted with buffer, pelleted (15,000 × g, 30 min), and stored at −80 °C in the above noted buffer containing 25% glycerol. All of the above steps were performed at 4 °C.

Binding and Cross-linking of Radiolabeled Glucagon to Membranes—[125I]iodo-Tyr10-glucagon and [125I]iodo-Tyr11-glucagon were prepared as described previously (21). All binding reactions were carried out in a buffer containing 90 mM Hepes, 100 mM NaCl, and 10 mM MgCl2 at pH 7.4. Stored membranes were diluted with the Hepes buffer, pelleted (2000 × g for 30 min), and resuspended in the buffer prior to use. Typically, 3 mg (wet weight) of membranes were

are processed and changes in glucagon-receptor structure are propagated at the level of the plasma membrane. We undertook these studies to investigate the structures of glucagon-receptor complexes that could be separately assigned to the high and low affinity binding sites that are characteristic of canine hepatic plasma membranes. While we readily identified an Mf = 54,000 protein to which radiolabeled glucagon could be cross-linked by use of 1,4-difluoro-2,5-dinitrobenzene (DFDNB)1 or bis(sulfosuccinimidyl)suberate (BS3), labeling of the protein was relatively insensitive to the presence of both GTP (a well recognized modulator of glucagon-receptor interactions, Refs. 1–6, 19) and competing hormone. In contrast, we discovered that upon high affinity GTP-sensitive binding, glucagon is processed by a membrane-associated and receptor-linked protease to a hormone fragment that remains in stable association with membranes even in the absence of cross-linker.

The abbreviations used are: DFDNB, 1,4-difluoro-2,5-dinitrobenzene; BS3, bis(sulfosuccinimidyl)suberate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEMED, N,N,N′,N′-tetramethylethylenediamine.
incubated with 100,000–200,000 cpm (0.06–0.12 pmol) of radiolabeled glucagon and other compounds such as GTP or glucagon in a total volume of 1 ml. The mixtures were incubated at 26 °C for 30 min to allow binding to reach equilibrium and were then either centrifuged at 2000 \( X_g \) for 30 min or subjected to cross-linking (2 mM DFDNB or 0.2 mM BS3) for 30 min at 20 °C prior to centrifugation. Analysis consisted of either counting the pellets for radioactivity by use of a Packard \( \gamma \) scintillation spectrometer or subjecting them to SDS-PAGE and autoradiography.

**RESULTS AND DISCUSSION**

Our initial approach to examining the state of membrane-bound glucagon relied on the use of chemical cross-linkers to identify membrane proteins to which radiolabeled hormone had been bound and on SDS-PAGE followed by autoradiography to separate labeled proteins. As shown by the autoradiograms of fixed gels in Fig. 1a, both DFDNB and BS3 were effective in cross-linking \( [[^{125}I]iodo-Tyr^1]glucagon \) to a protein with \( M_r = 54,000 \). (Although complexes cross-linked by DFDNB and BS3 differ slightly in electrophoretic mobility, these agents are well known to cause different extents of intramolecular cross-linking and thus slight alterations in protein conformation and in migration during SDS-PAGE conditions.) The results of Fig. 1a are consistent with previous studies identifying the association of glucagon with a membrane protein having \( M_r = 60,000 \) (Ref. 23, cf. Refs. 15–18).

Fig. 1b shows, however, that when gels were dried and subjected to autoradiography directly (that is, without prior fixation) a major band (apparent \( M_r = 30,000 \), hereafter called the lower molecular weight band, was evident in addition to the \( M_r = 54,000 \) band. Whereas a comparison of Fig. 1, a and b, suggests that the material which corresponds to the lower molecular weight band diffuses from the gel during fixation, Fig. 1c shows that the material is not present in our preparation of radiolabeled glucagon and that its appearance in no way depends on the addition of a cross-linking agent. Additional autoradiographic bands corresponding to peptides having apparent molecular weights lower than 30,000 are clear in the overexposed gels shown in Fig. 1 but actually represent material resulting from only a minor fraction of membrane-associated \( ^{125}I \)-labeled glucagon-derived peptides (see also Figs. 4 and 5).

**Fig. 1.** Analysis of membrane-associated material resulting from incubation of canine hepatic plasma membranes with radiolabeled glucagon. Membrane samples were incubated under selected conditions, and membrane-associated material was analyzed by SDS-PAGE followed by autoradiography. a, membranes incubated with \( [[^{125}I]iodo-Tyr^1]glucagon \), cross-linked with 0.2 mM BS3 (lane 1) or with 2 mM DFDNB (lane 2), and subjected to electrophoresis; the gel was fixed and stained prior to further analysis, the gels were then dried directly and exposed to x-ray film for 16–40 h at \(-80^\circ \)C. While representative data are presented, each experiment was repeated on at least two occasions with identical results. Additional controls shown in Fig. 1d identify the fact that, while the \( M_r = 54,000 \) band (representing \( ^{125}I \)-labeled glucagon cross-linked to receptor) all but disappears when membranes are washed prior to the addition of cross-linker (as the result of the dissociation of ligand), the lower molecular weight band remains intense. It is thus the case that the lower molecular weight band represents material in very stable association with membranes. The results of Fig. 1, e and f (illustrating experiments performed in the absence or presence of cross-linker, respectively), show that quite different patterns are obtained by the use of \( ^{125}I \)-labeled glucagon containing iodo-tyrosine at residue 10 or residue 12. While both radiolabeled ligands are readily cross-linked to the \( M_r = 54,000 \) receptor, only the former gives rise to the lower molecular weight material. Overall, this result suggests either that the formation of the lower molecular weight material is critically dependent on ligand structure or that it contains only the NH\(_2\)-terminal portion of the glucagon molecule.

Material eluted from the lower molecular weight band subsequent to electrophoresis migrated to its original position during SDS-PAGE and appeared in the included volume during gel filtration on Bio-Gel P-30. Fig. 2 shows that the elution volume of the lower molecular weight material from Bio-Gel P-2 was very nearly the same as that of the dodecapeptide glucagon(1–12), a result suggesting that material in the lower molecular weight band might correspond to a fragment of glucagon. Additional experiments examined the behavior of the material in the lower molecular weight band during reverse-phase high performance liquid chromatography. Fig. 3 shows that the radiolabeled material and \( [[^{125}I]iodo-Tyr^1]glucagon \) (lane 1–2) were separated on the reverse-phase column, and as important, that treatment of the material corresponding to the lower molecular weight band with either trypsin or carboxypeptidase A produced a peptide that eluted at a position indistinguishable from that taken by the NH\(_2\)-terminal dodecapeptide of radiolabeled glucagon. Finally (as expected from the fact that material in the lower molecular weight band can be converted to a peptide having the properties of \( [[^{125}I]iodo-Tyr^1]glucagon \) (1–12) by digestion with trypsin), treatment of the gel-filtered material by manual Edman degradation (26) revealed the release of iodo-tyrosine-derived radioactivity subsequent to the 10th cycle of cleavage (data not shown). It should be noted that only a few femtomoles of radiolabeled material were available for analysis by these procedures and that chemical sequence determination was not possible.

Results presented above provide a basis for considering the
structure of the material in the lower molecular weight band as a glucagon fragment. That is, the material (a) exhibits an apparent molecular size lower than that of $^{125}$I-labeled glucagon (as determined by gel filtration), (b) contains glucagon residues 1–10 (as determined by the identification of $^{125}$I-iodo-Tyr$^{7}$) at the 10th position during radiosequence analysis), (c) contains glucagon residues 1–12 and an unmodified NH$_2$-terminal glucagon domain (as determined by proteolytic processing of the material to $[^{125}$I]iodo-Tyr$^{10}$]glucagon(1–12)), and (d) contains a short COOH-terminal extension on the glucagon sequence 1–12 (as determined by the effects of both trypsin and carboxypeptidase A, enzymes that can act only COOH-terminal to Lys$^{7}$, to convert the material to $[^{125}$I]iodo-Tyr$^{7}$]glucagon(1–12)). Since carboxypeptidase A would not be expected to remove either Asp$^{19}$ or Arg$^{17}$ (both of which are distal to Lys$^{7}$ in the glucagon sequence), we conclude that material in the lower molecular weight band results from proteolytic cleavage of glucagon in the region Tyr$^{10}$/Lys$^{7}$. The lack of processing of $[^{125}$I]iodo-Tyr$^{10}$]glucagon to a related hormone fragment most probably arises from structural perturbation introduced by the iodine atom on Tyr$^{10}$. It is important to note that (presumably because of the failure of the relevant peptides to bind SDS with the usual stoichiometry) the membrane-bound glucagon fragment and authentic $[^{125}$I]iodo-Tyr$^{7}$]glucagon(1–12) ($M_r = 1500$) both migrate artifactualy during SDS-PAGE as if they were proteins with $M_r = 30,000$.
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...band (data not shown). We can thus say that the tightly membrane-bound glucagon fragment arises from a membrane-associated protease and from the previous interaction of intact radiolabeled glucagon with plasma membranes. Of a variety of protease inhibitors tested, only phenylmethylsulfonyl fluoride was effective at inhibiting the formation of the membrane-bound glucagon fragment (Fig. 4a), a result suggesting that the relevant enzymatic activity results from a serine protease and that the activity does not arise from any of several potentially contaminating lysosomal cathepsins. Most important, the formation of the fragment appears to be specific for the structure of glucagon; Fig. 4b shows that, while the formation of the fragment is inhibited by the addition of glucagon to incubations containing membranes and $^{125}$I-labeled glucagon, it is unaffected by the addition of the glucagon homologs secretin and vasoactive intestinal peptide or by the addition of arginine vasopressin.

Since the pattern of specificity identified above for the generation of the glucagon fragment is no different from that found for the cross-linking of radiolabeled glucagon to the $M_r = 54,000$ receptor (Fig. 4b), it appeared probable that the formation of the fragment and ligand binding to receptor might somehow be linked. Fig. 5 assesses in detail the association of $[^{125}]$jodo-Tyr$^{29}$]glucagon with receptor and the generation of the radiolabeled glucagon fragment, each as modified by the presence of glucagon and GTP. As illustrated by Fig. 5, a and b, respectively, the curves describing the inhibition of $^{125}$I-labeled glucagon binding to plasma membranes by glucagon and by GTP exhibit notable complexity, complexity resulting from high and low binding affinity components in the first case, and from GTP-sensitive and GTP-insensitive binding components in the second (1-6, 19). Our analysis of membrane-associated radiolabeled ligand under these two sets of circumstances (insets to Fig. 5, a and b) revealed that, while the $M_r = 54,000$ band (representing radiolabeled glucagon cross-linked to receptor) is relatively insensitive to the presence of glucagon or GTP (and can thus be considered to represent glucagon in association with low affinity GTP-insensitive receptors), the lower molecular weight band (representing the tightly bound uncross-linked glucagon fragment) shows considerable sensitivity to the presence of both effectors. Quantitative data presented in Fig. 5, c and d, clarify these relationships. In the case of inhibition by glucagon, we can identify the glucagon fragment as having arisen specifically from the high affinity component of glucagon binding to plasma membranes. In the case of inhibition by GTP, we can identify the fragment as having arisen specifically from the GTP-sensitive component of glucagon binding. Taken together, our results identify a link between the high affinity GTP-sensitive interaction of glucagon with hepatic plasma membranes and the proteolytic cleavage of the ligand to a glucagon fragment containing at least residues 1-13. While we cannot exclude the possibility that the formation of the fragment is related to a novel high affinity and GTP-sensitive interaction which does not directly involve the hormone receptor, our findings provide evidence for (a) the linkage of proteolytic activity to receptor binding by use of purified plasma membranes, (b) a heretofore unrecognized receptor-linked enzymatic activity of the glucagon receptor system, (c) the existence of separate physical and biochemical states of glucagon receptors which differ in their affinities for ligand, in their GTP sensitivities, and in their functional linkage to protease, and (d) the surprising avidity of the glucagon fragment for its binding site once it has been formed by receptor-mediated processes. They provide, in addition, a biological context for the demonstration by Balage et al. (27) that about 20% of radiolabeled glucagon bound to hepatic plasma membranes subsequently is found to have a decreased apparent molecular weight as assessed by gel filtration.

Although it remains to be seen whether the membrane-associated glucagon fragment described here plays a direct role in regulating the biological activity of the parent hormone, it is of interest that a COOH-terminal fragment of glucagon (a fragment which could arise from receptor-linked proteolysis of ligand) has recently been shown to inhibit the Ca$^{2+}$ pump of hepatic plasma membranes (28). More generally, the tight association of the glucagon fragment with plasma membranes would suggest that fragments of peptide hormones (peptides which might themselves have little ability to bind to hormone receptors) could well play roles in modulating hormone action, in emptying ligand from hormone-
whether the protease and the glucagon receptor are actually concerning how the protease proposed here and the glucagon receptor are physically linked, the mechanisms by which the fragment becomes so tightly membrane associated, or indeed, whether the protease and the glucagon receptor are actually one and the same remain to be investigated.

REFERENCES
Receptor-linked Proteolysis of Membrane-bound Glucagon