INSULIN DEGRADATION
X. IDENTIFICATION OF INSULIN DEGRADING ACTIVITY OF RAT LIVER PLASMA MEMBRANE AS GLUTATHIONE-INSULIN TRANSHYDROGENASE

PARTAB T. VARANDANI
With technical assistance of Mary Ann Nafz and Lois A. Shroyer
The Fels Research Institute, Yellow Springs, Ohio 45387 (U.S.A.)

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SUMMARY. The liver plasma membrane preparation devised by Neville (Biochim. Biophys. Acta, 154, 540 (1968)) contains insulin-degrading activity. Examination by chromatography on Sephadex G-75 of the products formed from ^{125}I-insulin upon incubation with plasma membrane showed the same products (A chain and B chain rich-A chain aggregate) as previously found with purified GSH-insulin transhydrogenase (GIT). In Ouchterlony double-diffusion experiments with antibody to purified rat liver GIT, plasma membrane gave a single precipitation band of identity with purified rat liver GIT. Thus, the insulin-degrading activity present in the plasma membrane preparation is indeed GIT.

There have been several reports of the presence of insulin-binding activity ("insulin-receptor" protein) in the plasma membrane fraction of liver; the same workers have also observed insulin-degrading activity in these preparations. However, it has been believed that insulin binding and degradation represent independent processes (for a recent review, see ref. (1)).

Glutathione-insulin transhydrogenase (GIT, thiol: protein disulfide oxidoreductase, EC 1.8.4.2) catalyzes the degradation of insulin by splitting the hormone at the disulfide bonds (2-6). The current study was undertaken to determine whether the insulin-degrading activity present in the plasma membranes is that of GIT. We isolated the plasma membrane fraction in accordance with the procedure of Neville (7); we chose this procedure because most of the insulin-receptor studies have used it or a slight modification thereof. The results show that the insulin-degrading activity present in the Neville preparation of rat liver plasma membranes is indeed GIT.

METHODS. Membrane purification from 30 gm of rat liver (homogenized in 1 mM $NaHCO_3$) was carried through step 11 according to Neville (7). The membrane float (about 36 mg of protein) was suspended in 6 ml of 1 mM $NaHCO_3$ and purified on a discontinuous sucrose density gradient made by layering 1.5 ml of 50% sucrose, 6 ml of 41% sucrose, 6 ml of 37% sucrose, and 1 ml of the membrane float. The gradients were centrifuged at 91,000 g for 120 minutes in the SW-27.1 rotor (Beckman Instrument Co.)). After centrifugation, the band at the 41%:37% sucrose

interface was removed with a pasteur pipette, left overnight at -15°, and then diluted 1:5 with water. The solution was centrifuged at 91,000 g for 30 minutes; the sedimented membrane was finally resuspended in 2 ml of 0.25 M sucrose-50 mM tris, pH 7.5 buffer (~ 6 mg protein/ml) and used for further studies.

The microsomal fraction was prepared as described previously (8) except that the homogenization of liver in 0.25 M sucrose-0.05 M tris-HCl (pH 7.5) was carried out with the use of a Dounce homogenizer in the manner described by Neville (7) rather than with a Potter-Elvehjem homogenizer.

Insulin-degrading activity was measured essentially as described previously (8) by the conversion of ^{125}I-insulin to a form soluble in 5% trichloroacetic acid. A mixture of ^{125}I-labeled and unlabeled insulin (0.10 μM) and 1 mM GSH was incubated at 37° in a total volume of 1 ml of 0.1 M potassium phosphate-5 mM EDTA-0.3% bovine serum albumin (final pH 7.5) with several levels of plasma membrane protein (0.1 to 0.7 mg). After ten minutes, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The precipitates were collected by centrifugation and washed as previously described. The rates of insulin degradation reported below have been corrected for degradation occurring in control incubations with plasma membrane omitted. All calculations are based on the portion of the reaction showing zero order kinetics. One unit of GIT activity is arbitrarily defined as the net degradation of one percent of the insulin present in 10 minutes under the conditions described.

The nature of the degradation products was studied by chromatography on Sephadex G-75 as reported previously (9, also see 10,11); the procedure is described in the legends to the figures.

5'-Nucleotidase was assayed by the method of Song and Bodansky (12). Procedures for the assay of glucose-6-phosphatase, succinate-INT reductase, and protein, Ouchterlony double-immunodiffusion, and the preparation of purified rat liver GIT, rabbit antibodies to GIT, and ^{125}I-labeled insulin have been described (8). The purified ^{125}I-insulin (~0.6 atom of iodine per molecule) used showed > 95% binding with excess insulin antibodies.

RESULTS. The purity of the membrane preparations was checked by the assay of marker enzymes. The specific activity of the plasma membrane marker enzyme 5'-nucleotidase was approximately 12 times higher in the plasma membrane preparations than in the initial homogenate. The specific activities of glucose-6-phosphatase and succinate-INT reductase (a marker for mitochondria) decreased to 1/4 and 1/8, respectively, in the plasma membranes relative to the initial homogenate. The magnitudes of these changes are similar to those reported by Pohl *et al* (13).

The dependence of insulin degradation on the amount of plasma membrane protein is shown in Figure 1. The specific activity of the insulin-degrading activity of the plasma membrane preparations amounted to 38± 12 units per mg of protein in 6 experiments. The specific activity of the initial homogenate was approximately 7 times higher than that of the plasma membranes. These values were obtained in the presence of 1 mM GSH: ommision of GSH decreased the rate of insulin degradation by the plasma membrane by one third. The addition of

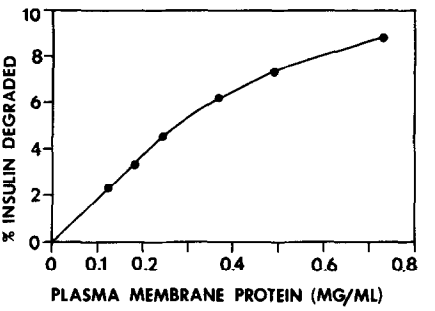

Figure 1. Insulin degradation by plasma membranes as a function of protein in the presence of 1 mM GSH. Assay conditions are described in text. All values are corrected for non-enzymatic degradation.

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15 mM N-ethylmaleimide, a sulfhydryl blocking agent, abolished all insulin-degradation. All of these observations are consistent with those reported by Freychet et al (14) for the plasma membrane insulin-degrading activity.

The specific activity of the Triton X-100 untreated and treated microsomal-bound insulin-degrading activity was respectively 4 and 8 times higher than that of the plasma membrane enzyme. Triton treatment had no effect on the plasma membrane insulin-degrading activity; thus, contrary to the situation in the microsomal fraction where most of the GIT is present in a latent state (8), the enzyme is fully active in the plasma membrane.

In double-immunodiffusion experiments with antibody to purified rat liver GIT (Fig. 2), both the partially (membrane float) and fully purified plasma membrane preparations showed a single precipitation band which was continuous with the precipitation band of purified GIT and also that of the starting homogenate. These results indicate that plasma membranes contain a protein immunologically identical to GIT.

Insulin is degraded by a two-step sequential process (9-11), i.e. insulin is first split by GIT to A and B chains which are then proteolytically degraded to low molecular weight components. Since the rate of proteolysis of the intermediate product (A chain) is much less in medium containing EDTA, 1 nM ^{125}I-insulin was incubated with 0.255 mg/ml of plasma membrane protein in

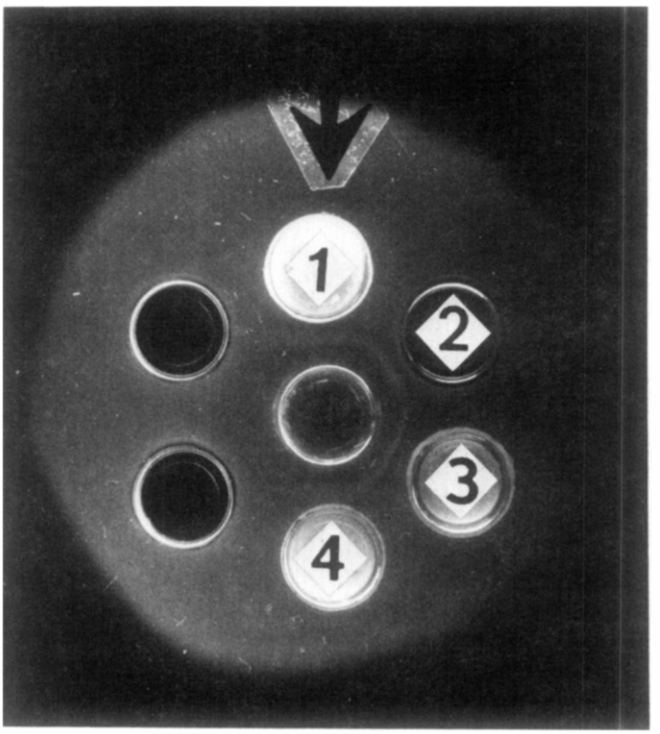
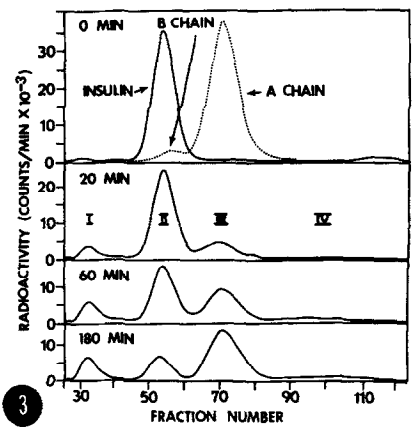


Figure 2. Ouchterlony double-immunodiffusion of plasma membranes with rabbit antiserum to purified rat liver GIT. The center well contained 60 µl antiserum. Each of the peripheral wells received 100 µl of: (1), membrane float (step 11, ref. 7) (0.51 mg protein); (2), 6 µg purified rat liver GIT; (3) starting homogenate (0.53 mg protein); and (4), plasma membrane (0.61 mg protein). The other peripheral wells were empty. Diffusion was carried out for 72 hours at 5°C.

phosphate buffers with and without EDTA (Figs. 3 and 4); no exogenous GSH was added in these experiments. Some representative profiles of radioactivity obtained by chromatography on Sephadex G-75 of the products of incubation in buffer containing EDTA are shown in Fig. 3. The amounts of radioactivity in various peaks for all time-periods of incubation and for both incubation media are presented in Fig. 4. In the EDTA-containing buffer, as the radioactivity in the insulin peak (designated Peak II) disappeared, only two major radioactive peaks, at the position of standard A chain (Peak III) and at the void volume of the column (Peak I), formed during the entire incubation period of 180 minutes; almost no radioactivity occured in the low molecular weight components (Peak IV). In the phosphate buffer without EDTA, the radio-



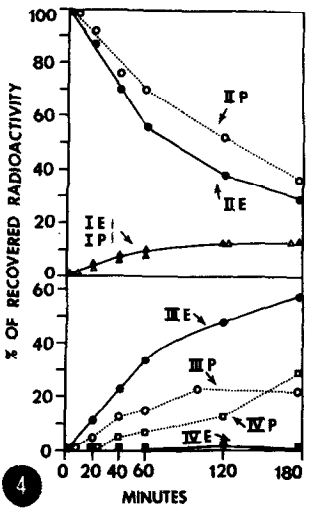


Figure 3. Radioactivity profiles on a Sephadex G-75 column of 1 nM ^{125}I-insulin that had been incubated for different time periods with 0.255 mg protein of plasma membrane per ml of 0.1 M potassium phosphate-5 mM EDTA buffer (pH 7.5) containing 0.3% bovine serum albumin. Incubations were terminated by the addition of N-ethylmaleimide solution (15 mM final concentration). Solid urea (1.44 g) and 40 μl of a mixture of unlabeled S-sulfonated A chain (40 μg) and B chain (60 μg) as carrier were added to the incubation mixture, the volume was brought to 6 ml with glacial acetic acid, and the entire mixture was then applied to a 2 cm x 45 cm Sephadex G-75 column equilibrated with 50% acetic acid. The column was eluted with 50% acetic acid at the rate of 1 drop/ 3 seconds;100-drop (1.45 ml) fractions were collected. Profiles for a few selected time periods are shown for illustration; the data for all time periods are shown in Fig. 4. In the upper panel the radioactivity pattern of ^{125}I-labeled insulin that had been subjected to sulfitolysis is shown (••••••••••••); 90% of the radioactivity of the original insulin was in A chain and 10% in B chain.

Figure 4. Amounts of radioactivity in different peaks of Sephadex G-75 chromatograms of 1 nM ^{125}I-insulin incubated in phosphate buffer with (——————) and without (••••••••••••) 5 mM EDTA for different time periods as described in the legend of Fig. 3. Radioactivity in the fractions under the peaks was numerically integrated, and the activity in each peak was expressed as a percentage of the activity recovered from the column; the recoveries of radioactivity from the column were nearly quantitative. Roman numerals denote peak numbers (I, B chain rich-A chain aggregate; II, insulin; III, A chain; IV, low molecular weight components). "E", incubation in buffer containing EDTA; "P", incubation in buffer without EDTA.

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activity first appeared in Peak III and Peak I; after 20 minutes radioactivity began to appear in the low molecular weight component (Peak IV). Peak III has been identified by several criteria as the A chain of insulin and Peak I has been shown to be an aggregate of A and B chains (linked through disulfide

bonds) with the ratio of B chain to A chain much greater than one (10). Purified GIT has also been found (2-6, 15) to yield the same products (i.e., A chain, and B chain rich-A chain aggregate).

DISCUSSION. In agreement with other workers (1), we found that rat liver plasma membranes obtained by the Neville procedure contain an insulin-degrading activity Immunologic data and the nature of the degradation products of insulin clearly establish that the insulin-degrading activity in plasma membranes is due to the presence of GIT.

Thus, the insulin degrading enzyme, GIT, as well as the protease(s) which further degrade the products formed by GIT, are present in the liver plasma membrane. Freychet et al (14) were unable to detect A or B chain when they incubated 0.18 nM ^{125}I-insulin with 1.9 mg per ml of liver membranes for 90 minutes. As fully discussed elsewhere (9; cf. 10,11), for the accumulation of the product of the first reaction of insulin degradation (i.e., that catalyzed by GIT) it is necessary either (a) to use a high insulin/tissue ratio (by raising the concentration of insulin markedly or reducing the amount of tissue protein), or (b) to inhibit the activity of the second proteolytic stage of degradation by the addition of competitive substrates (e.g. A and B chains of insulin) or an inhibitor such as EDTA. So Freychet et al apparently failed to observe the formation of A or B chains because they used far too much membrane protein (7.5x that used in the current study) relative to their insulin concentration (1/5 that of the current study).

We reported recently (8) that rat liver GIT is located primarily in the membranous component of the microsomal fraction; it is present in the lipoprotein residue of the microsomal membrane. The finding of markedly lower specific activity of GIT in the plasma membrane as compared to the microsomal fraction raises the question as to whether GIT is an intrinsic component of plasma membranes or is present due to the contamination with microsomal elements. We are unable to answer this question with certainty since this study was designed only to characterize the plasma membrane insulin-degrading activity re-

ported by others. However, since the plasma membranes retained GIT activity even after resedimentation, GIT appears to be intrinsic to the plasma membranes. In addition, the occurrence of GIT in the plasma membranes is consistent with the following characteristics of GIT: (a) The action of GIT is the first and rate-controlling step of insulin degradation in all the 5 rat tissues examined (9-11). (b) Hepatic GIT activity in the rat is under a feedback control system by insulin (16). (c) GIT is ubiquitous (17). This is in keeping with the known multifunctional and widespread nature of the actions of insulin. (d) There is evidence that GIT and insulin-receptor proteins probably co-exist. As discussed elsewhere (8), treatment of the liver microsomal fraction with Triton X-100 or phospholipase A or C results in unmasking and solubilization of both the GIT and insulin-binding activities. Other recent work from this laboratory shows that GIT occurs in the leukocyte fraction of human blood (18). Since GIT is also present in thymus (17), it seems probable that lymphocytes contain GIT. Lymphocytes have been shown to contain insulin-binding activity(19). This again indicates that the two proteins probably co-exist.

Emmelot et al (20) have demonstrated that numerous enzyme activities occur both in isolated plasma membranes and in microsomes. While the specific activities of some of these enzymes (e.g.,Mg^{2+}-ATPase, Na^{+}-K^{+}-Mg^{2+}-ATPase, 5'-nucleotidase and NAD pyrophosphatase) were found to be much greater in the plasma membranes than in the microsomal preparations, others (e.g., glucose-6-phosphatase, $NADH_2$-cytochrome C reductase and NAD nucleotidase) were found to be 4-9 x higher in the microsomes than in the plasma membrane preparations; GIT activity would belong to this latter group. These authors (20) are of the opinion that the presence of small amounts of enzymes in the latter group do not necessarily represent microsomal contamination, but rather may indicate a functional continuity between the two membrane systems. The membranes seem to be biogenically related (21) and are believed to be parts of a continuum, i.e., membrane is initially produced in the endoplasmic reticulum, transported to (and modified in) the Golgi complex, and finally becomes part of the plasmalemma. Thus, GIT

in the microsomal fraction could represent a storage form of GIT activity, with only the very small fraction of the total cellular content (in the cell membrane) necessary for its physiological function (cf. 22,23).

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