Rapid Publication

Human Proinsulin-Specific Antigenic Determinants Identified by Monoclonal Antibodies

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SUMMARY

Antigenic determinants recognized by human proinsulin (HPI)-specific monoclonal antibodies (Mabs) and Mabs crossreacting with free human C-peptide (HCP) were mapped by using various forms of purified, partially converted HPI intermediates. Two HPI-specific mouse Mabs (GS-4G9 and GS-9A8) reacted with the same antigenic determinant, GS, which was localized to the site of linkage of the B-chain to the C-peptide (Arg-Arg) at positions 31–32. These antibodies bind with equal efficiency to C65-A1 split proinsulin and to intact HPI. The binding of C32-C33 split proinsulin is markedly reduced.

A rat Mab (GN-VIIB6), which crossreacts with free HCP in addition to HPI, reacted similarly with various HPI intermediates as it had with the corresponding synthetic HCP fragments, as previously reported (see ref. 9). This determinant (GN) is a three-dimensional structure composed of residues located in two separate regions in the C-peptide segment (positions 40–45 and 57–63). Reduced, carboxymethylated HPI retains the GN-determinant, whereas all insulin-like immunoreactivity identified with a conventional guinea pig insulin antiserum is completely lost. The binding of the two GS Mabs to the denatured HPI was reduced by 40–50% compared with intact HPI.

It is concluded that the strong GN-determinant can readily form in the C-peptide segment of HPI, independently of the presence of ordered structure in the insulin moiety. A predicted β-turn at position 47–50 may play an important role in bringing N- and C-terminal regions of the C-peptide segment into close proximity. The spontaneous formation of the GN-determinant could thus be a crucial step in the formation of the correct disulfide bonds in more distant parts of the newly synthesized HPI-polypeptide chain. **DIABETES 1984; 33:1012–16.**

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Received for publication 15 June 1984.

Proinsulin[1] is converted to insulin by excision of the central C-peptide segment connecting the B- and A-chains. Conversion is initiated in the Golgi apparatus, and continues into the maturing secretory granule.[2] Insulin and C-peptide are co-secreted from the pancreatic β-cell in equimolar amounts.[3] The proteolytic cleavage sites in HPI appear to be the two dibasic residue pairs, Arg-Arg (position 31–32) and Lys-Arg (position 64–65), flanking the C-peptide segment. Complete processing involves the removal of all four basic residues.[4] However, a significant amount (~6%) of the proinsulin molecules escape the processing machinery either completely or partially, leading to the co-secretion of intact proinsulin and both intermediate forms in which the A–C or B–C junctions have been processed together with insulin and C-peptide.[5-8]

The establishment of two groups of hybridomas (GS and GN) secreting Mabs reacting either specifically to the prohormone HPI (GS) or crossreacting with free HCP (GN)[9] and the availability of a series of purified and characterized HPI intermediates allowed us to further characterize and map out these two antigenic determinants in the HPI molecule.

MATERIALS AND METHODS

Antibodies. The HCP-directed rat monoclonal antibody (Mab) GN-VIIB6 (IgG2a, tissue culture supernatant) and the HPI-specific mouse Mabs GS-4G9 and GS-9A8 (IgG2b and IgG1, ascites fluid) were derived[9] and purified[10] as described. Guinea pig antiserum to human insulin (GpI) was from DAKO (Accurate Chemicals, Westbury, New York).

Antigens. Biosynthetic human proinsulin (HPI) and human C-peptide were prepared and characterized as described.[11] Human insulin was prepared as described.[12]

Reduced and carboxymethylated HPI was prepared by the method of Moore and Stein.[13] Monoiodinated (I^{125}) A-14-

TABLE 1
Crossreactivity of antibodies to various processed forms of proinsulin

Antigen / Antibody	HPI	HPI split 65166	HPI split 56157	HPI split 32133	HPI des 33-56	HCP	Insulin	Denatured(RCM) HPI
HPI-specific GS-4G9	1	0.88 ± 0.15	0.96 ± 0.15	<0.01	<0.01	<0.03	<0.01	0.62 ± 0.07
HPI-specific GS-9A8	1	1.08 ± 0.09	0.94 ± 0.07	0.02 ± 0.01*	<0.001	<0.003	<0.001	0.53 ± 0.09
HCP-directed GN-VIIB6	1	0.84 ± 0.12	0.43 ± 0.08	0.77 ± 0.13	0.03 ± 0.01*	1.20 ± 0.19	<0.002	0.88 ± 0.05
Insulin antiserum GpI	1	1.53 ± 0.20	1.25 ± 0.11	1.48 ± 0.14	1.21 ± 0.15	$<3 \times 10^{-5}$	2.10 ± 0.25	<0.001

The crossreactive parameters were calculated as described in MATERIALS AND METHODS. The values are mean ± SD from duplicate or replicate determinations. *I_{20} has been used for these calculations. "Less than" values indicate that the highest antigen concentration did not displace binding. These values were calculated by using the I_{10} value for HPI divided by the maximal concentration used of the respective peptide. The concentration range of competing peptides tested depends on the affinity constants of the antibodies toward intact HPI. For the three monoclonal antibodies the concentration range tested was $2 \times 10^{-10} - 2 \times 10^{-7}$ M, representing the entire displacement curve for intact HPI. The corresponding range for GpI (having the higher avidity) was $2 \times 10^{-12} - 2 \times 10^{-9}$ M. The concentration of tracer (monoiodinated HPI) was approximately 10^{-11} M.
In the small figures indicating the conformation of the various intermediates, the open, black, and dotted structures represent A, B, and C segments, respectively. The big bars (open and solid) in the insulin portion indicate α-helical regions, and are based on x-ray crystallographic analysis[16,17] and on the apparent conservation of the structure of the insulin moiety in proinsulin.[18-20] The small bars indicate the dibasic residues at conversion sites. The stippled bars in HCP could indicate some kind of ordered structure in these regions;[21,22] however, no clearcut evidence on its nature has been presented.[18]

human proinsulin was prepared with a specific activity of ~210 μCi/μg as previously described.[14]

The HPI intermediates were prepared by limited enzymatic hydrolysis, isolated using preparative high-pressure liquid chromatography methods, and then characterized by amino acid analysis and amino acid sequencing (Frank and Pekar, manuscript in preparation).

Equilibrium binding assay. Solution-phase equilibrium antibody binding assays were carried out as described elsewhere.[9,15] The following modifications were made: final incubation volume was 250 μl per tube (RIA vial no. 73, 1055 in styrofoam racks no. 95, 1046, Sarstedt, Princeton, New Jersey), where 100 μl tracer (^{125}I-HPI, 5000 cpm/tube), 50 μl sample (competing peptide), and 100 μl diluted antibody were added in the sequence indicated using phosphate-buffered saline pH 7.2 as solvent supplemented with 0.5% bovine serum albumin (Miles, Elkhart, Indiana), 0.18% bovine gamma globulin (Sigma, St. Louis, Missouri), and Trasylol (200 U/ml from Bayer, Mobay Chemical Corp., New York, New York). Antibody precipitation by polyethylene glycol mol wt 8000 (Carbowax, PEG 8000, Fisher, Fair Lawn, New Jersey) was used to separated bound from free antigen. Racks were centrifuged using adaptors for microtiter plates, and aspiration of supernatants was done with a Wheaton manifold (no. 851381, Wheaton, Millville, New Jersey).

The various antibody preparations were used in the following dilutions, GN-VIIB6 1:800; GS-4G9 1:7000; GS-9A8 1:23,000; and GpI 1:110,000 to obtain a desired zero-dose binding of approximately 40% (i.e., the maximal precipitation of tracer obtained in the absence of competing peptide).

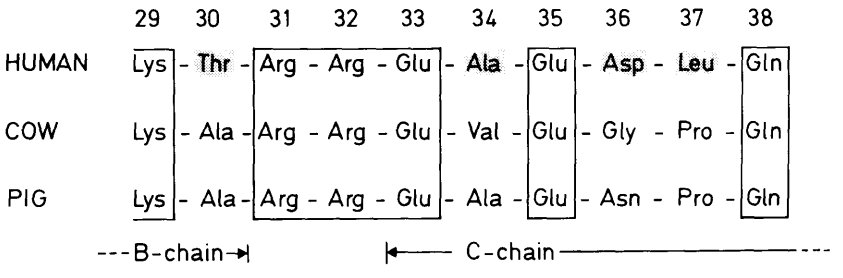

FIGURE 1. Localization of the GS-determinant. Comparison of the primary structure around the B-C junction of proinsulins in three species. The human sequence[23,24] expresses the GS-determinant, whereas the bovine[3,4] and porcine[25] sequences are unable to bind any of the GS-antibodies.[9] The shaded residues are probably the major contributors to the GS-determinant.

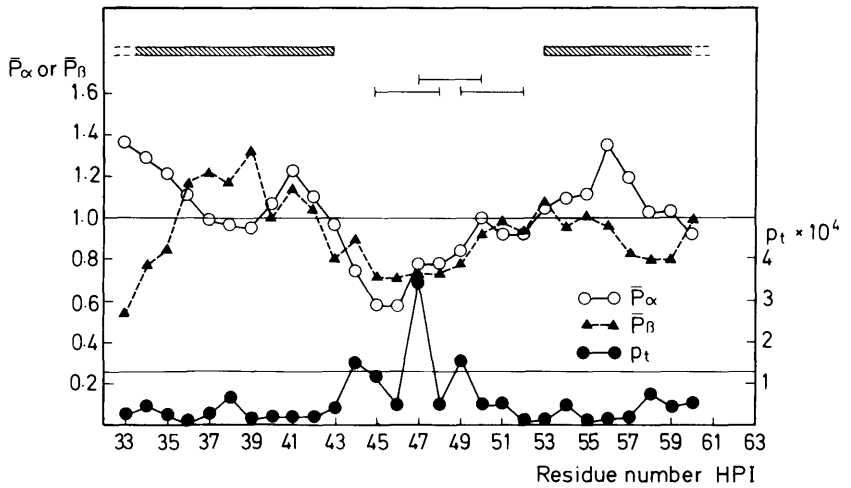

FIGURE 2. Predicted secondary structure of HCP. The Chou and Fasman[26,27] methods for predicting secondary structure were applied to the HCP sequence using a computer program developed by Dr. I. Hanukoglu.[28] Left ordinate: $\bar{P}_\alpha$ and $\bar{P}_\beta$ are the average conformational parameters of α-helical and β-sheet formation. Right ordinate: probability of β-turn ($p_t \times 10^4$). The lower horizontal line represents an arbitrary cut-off value of 1.25×10^{-4}. The hatched bars represent regions of possible α-helical or β-strand structure, and the brackets denote probable β-turns.

From displacement curves (plotting bound tracer/zero-dose binding versus competing peptide concentration) the concentration of the various antigens required to displace 50% (I_{50}) of antibody binding to ^{125}I-HPI was estimated. The crossreactivity to a particular Mab of any given antigen as compared with intact HPI is expressed as the ratio of I_{50} (HPI)/I_{50}. Thus values greater or smaller than one indicate higher or lower affinity toward the respective antigen relative to intact HPI.

RESULTS AND DISCUSSION

Crossreactivity of various modified forms of HPI (Table 1) was measured relative to that of intact HPI to the following four antibody preparations: (1) and (2) GS-4G9 and GS-9A8; two different mouse Mabs both showing a unique specificity toward HPI (affinity constants $K_a = 1.3 \times 10^8$ M^{-1} and 6.1×10^8 M^{-1}, respectively) in that neither crossreacts with HCP, insulin, or with bovine or porcine proinsulin;[9] (3) GN-VIIB6; a rat Mab (one of a series of 11 with identical specificity) that reacts with a complex (three-dimensional) determinant located in the HCP segment (affinity constant $K_a = 6.6 \times 10^8$ M^{-1}) and also expressed in free HCP;[9] (4) GpI; a conventional guinea pig antiserum raised against human insulin. The results (Table 1) clearly demonstrate that both GS antibodies react to a very similar determinant that is only expressed when the junction between B- and C-chains is intact. A single proteolytic cleavage at position 32–33 (C32–C33 split HPI) reduces the reactivity of the intermediate to a few percent. Two other intermediates (C65-A1 split HPI, C56–C57 split HPI) having the intact B-C junction display an unchanged reactivity compared with intact HPI toward both antibodies. The reactivity toward reduced and carboxymethylated HPI is slightly reduced (by 40–50%) for GS-4G9 and GS-9A8. It is concluded that the two GS-antibodies recognize very similar, if not identical, antigenic determinants. This is in full agreement with results previously reported from various antibody competition experiments[9] in which the two antibodies behaved identically. The sequence thought to contain the antigenic determinant GS in HPI is shown in Figure 1, and is compared with the nonreactive homologous regions in porcine and bovine proinsulins.

Interestingly, the two identically reacting antibodies derived from the same hybridoma fusion have different affinities toward HPI, and are of different IgG subclasses. A comparison of the primary structures of the hypervariable regions of the two antibodies could provide useful information on the structure of related paratopes (antigen binding sites).

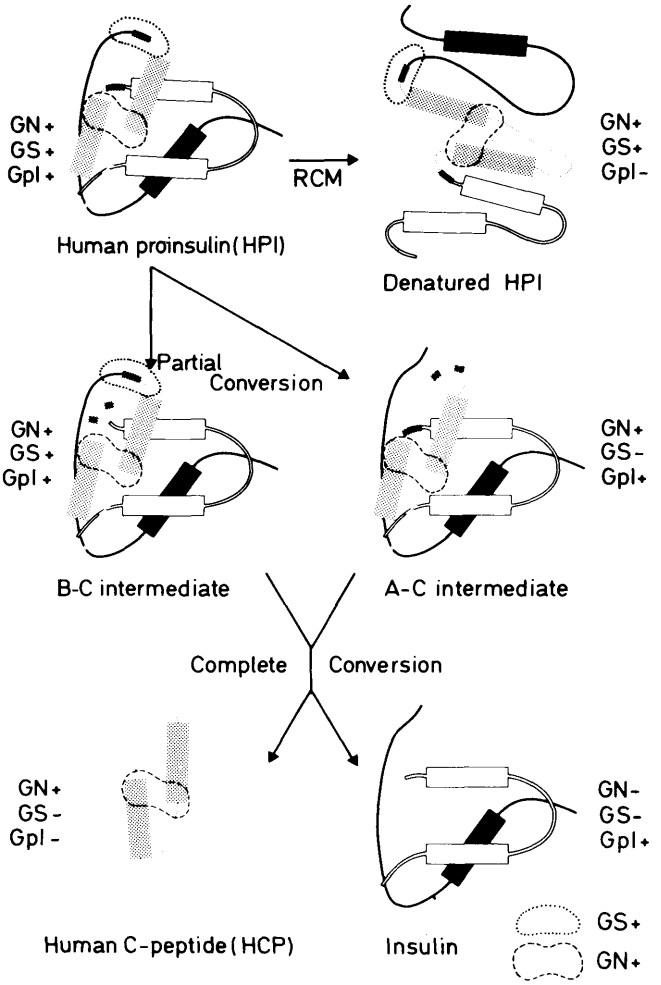

FIGURE 3. Summary of immunoreactivity of the products in the proinsulin processing pathway. RCM = reductive carboxymethylation of cysteines. (See Table 1 legend for details on the representation of HPI.)

The GN-determinant recognized by antibody GN-VIIB6 is fully expressed in all the HPI intermediates having an intact HCP-sequence (C65-A1 split HPI, C32–C33 split HPI and free HCP). The reactivity to the intermediate is reduced by a factor of 2 by a single proteolytic cleavage in the HCP segment (C56–C57 split HPI) that separates the two regions forming the intact determinant (Table 1). The binding to this intermediate is comparable to that of the synthetic HCP fragment representing the HPI positions 44–56, as previously reported[9] (data not shown).

Interestingly, the GN-determinant is fully intact in reduced and carboxymethylated HPI. This denaturation process involving the breaking of the three S-S bonds in the insulin moiety (7–72, 19–85, 71–76) totally destroys all insulin-like reactivity as identified by the polyclonal insulin antiserum GpI (Table 1). All the intermediates with an intact insulin moiety have an increased reaction with GpI, and maximal reactivity is obtained with mature insulin, which is twice as efficient as HPI in displacing the antibody-bound tracer (Table 1).

The formation of the intact GN-determinant requires approximately 75% of the HCP-sequence (position 40–63). The binding site appears to be made up of residues within the regions 40–45 and 57–63,[9] which may be brought together into a specific structure by the flexible glycine-rich central portion of the connecting segment 46–56. Chou and Fasman analysis[26,27] to predict secondary structure indicates the presence of a β-turn (Figure 2) located at positions 47–50 (Gly-Pro-Gly-Ala) in HCP, as proposed by Snell and Smyth.[29] This β-turn could bring the two immediate flanking regions together (in either α-helical or β-strand configuration) to form the intact GN-determinant. As shown, this folding can occur in free HCP, as well as in HPI, independently of the presence of the correct disulfide bridges in the insulin moiety. It is therefore tempting to speculate that the β-turn plays a key role in the formation of the mature structure of HPI. The folding of the correct three-dimensional GN-determinant may subsequently mediate the correct formation of disulfide bridges in the more distant regions of the HPI molecule. Interestingly, the prediction of a conserved central β-turn in the C-peptide segment in several species has been reported.[26,29]

A summary of the expression of particular antigenic determinants on HPI during the proteolytic maturation process is shown in Figure 3. Although the efficient expression of the GN-determinant in both denatured and intact HPI as well as in HCP might suggest that the free HCP retains a conserved structure in solution, a more detailed kinetic analysis will be necessary to decide this issue. However, recent studies of peptide structure carried out by generating protein-reactive monoclonal antibodies against short derived synthetic peptides[30] have shown that the frequency with which such antibodies exhibit full crossreactivity to both the peptide and the native protein is much too high to favor the general assumption that peptides adopt many random conformations in solution.[31] A likely explanation could be that some peptides favor particular conformation(s) that are similar to that in the domain structure of the parent protein.

The results obtained with reduced and carboxymethylated HPI suggest that both types of monoclonal antibodies would be especially useful in combination with insulin antisera to study proinsulin biosynthesis at the EM-level. Presumably, the GN- and GS-determinants would be expressed in newly synthesized HPI-molecules that are still not folded and oxidized into the mature structure with insulin-like immunoreactivity. Even earlier stages such as the nascent polypeptide chain still complexed to the ribosomes might be recognized by the GS- and GN-antibodies, provided the residues specifying the determinants were translated.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Israel Hanukoglu for computer-assisted prediction of HCP secondary structural features, Drs. Arthur H. Rubenstein and Robert M. Cohen for helpful discussions, and Cathy Christopherson for her skillful assistance in preparing this manuscript.

This work was supported in part by USPHS Grants AM 13914 and AM 20595.

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