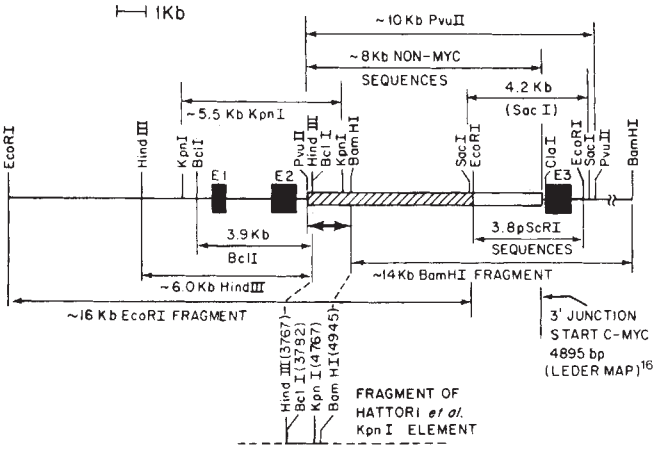

Fig. 4 Structural model of rearranged *myc* locus containing an inserted L1 element. The diagram shows a blocked area of ~8 kb of non-*myc* sequences inserted between *myc* coding exons 2 and 3, and is based upon preliminary Southern data as explained in the text. The open portion of the blocked area, labelled as 3.8pScR1, is described in the text. The cross-hatched portion of the blocked area represents the remainder of the non-*myc* sequences we have not yet cloned or analysed. The presumed origin of the lighter intensity, tumour-specific, rearranged *myc* bands (the 16 kb *Eco*RI, 10 kb *Pvu*II, 4.2 kb *Sac*I, 3.8 kb *Eco*RI, and 3.8 kb *Eco*RI/*Pvu*II bands) seen in Fig. 1*a* are shown in the model. Other restriction fragments sizes (the 14 kb *Bam*HI, 6 kb *Hin*dIII, 5.5 kb *Kpn*I, and 3.9 kb *Bcl*I fragments) have been seen on many Southern blots (data not shown) and have been used to map the 5′ end of the proposed insert. The partial restriction map of a fragment taken from the L1 element (numbered as in the original paper[8]) is provided for comparison with the restriction endonuclease pattern we determined in the 5′ region of the insert.

identified within the full-length L1 element[8]. Therefore, we postulate that the entire disrupting sequence is an L1 element which has inserted between *myc* coding exons 2 and 3. The relatively large size of this L1 may be a reflection of either the presence of novel L1 sequences, the existence of non-L1 sequences in the body of the element, or of internal reiterations. Alternatively, due to inherent errors in determining DNA molecular size using agarose gels, this element may in fact be smaller (in the normal range 6–7 kb). Cloning of the entire element will resolve these possibilities, and allow us to study the complete structure of this interesting L1 element.

We thank Drs Maxine Singer, Steven Potter, Jerry Freed and Alfred Knudson for criticisms of this manuscript, Dr Potter for a *Kpn*A clone, Dr Michael Erisman for *myc* probes, Dr Vinayaka Prasad, Dr Munir Khan and Vidya Jonnalagadda for discussions, Mark Shlomchik, Robert Kleinfield and Anita Cywinski for sequencing assistance, Rich Bomze, Willie Barr, Harold Ott, Karen Trush, Joe Hurley and Clara Brett for graphics work, Bob Stodola, Dr Frank Manion and Paul Marcolina for help in computer sequence analysis and Secretarial Services for preparation of this manuscript.

This work was supported by grants an Appropriation from the Commonwealth of Pennsylvania, P.G.R. was a Special Fellow of the Leukemia Society of America. The sequence data in this publication have been submitted to the EMBL/GenBank Libraries under the accession number Y00304.

Received 1 February; accepted 17 March 1988.

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Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*

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Immunoglobulin heavy-chain binding protein (BiP, GRP-78) associates tightly in the endoplasmic reticulum (ER) with newly synthesized proteins that are incompletely assembled, have mutant structures, or are incorrectly glycosylated[1–3]. The function of BiP has been suggested to be to prevent secretion of incorrectly folded or incompletely assembled proteins[2,3], to promote folding or assembly of proteins[3], or to solubilize protein aggregates within the ER lumen[4]. Here we examine the interaction of BiP with newly synthesized polypeptides in an *in vitro* protein translation–translocation system. We find that BiP forms tight complexes with nonglycosylated yeast invertase and incorrectly disulphide-bonded prolactin, but does not associate detectably with either glycosylated invertase or correctly disulphide-bonded prolactin. Moreover, BiP associates detectably only with completed chains of prolactin, not with chains undergoing synthesis. We conclude that BiP recognizes and binds with high affinity *in vitro* to aberrantly folded or aberrantly glycosylated polypeptides, but not to all nascent chains as they are folding.

Immunoglobulin heavy-chain binding protein (BiP) has been shown to be identical to the glucose regulated protein of relative molecular mass (M_r) 78,000 (GRP-78) and is a member of the evolutionarily conserved HSP-70 family of heat-shock related proteins[4]. Manipulations which inhibit N-linked glycosylation of proteins induce BiP synthesis[5,6]. Furthermore, BiP shows increased binding to immunoglobulin heavy chains[2], influenza haemagglutinin[3], and tissue plasminogen activator[7] when glycosylation is inhibited with tunicamycin. Exposure of cells to anoxia[8], amino acid analogues[9] and sulphydryl-reducing agents[10], which do not specifically affect glycoprotein synthesis, also induce BiP synthesis. In addition, BiP binds to mutant but correctly glycosylated proteins[3]. A plausible explanation of these observations is that BiP recognizes incorrectly folded proteins.

To examine whether BiP associates only with aberrant proteins or also plays a role in the folding of newly synthesized proteins in the ER, we used a coupled protein translation–translocation system consisting of a wheat germ translation extract supplemented with dog pancreas rough microsomes. Addition of messenger RNA that encodes a secreted protein leads to the efficient targeting and cotranslational translocation of the protein into the lumen of the microsomes[11]. As BiP is an abundant component of dog pancreas microsomes (C.K.K. and R.B.K., unpublished data), an interaction of BiP with newly synthesized proteins can be detected by precipitating BiP complexes from the lysed microsomes using a BiP-specific antibody.

Yeast invertase, a highly glycosylated protein, was used to examine the effects of altered glycosylation on association with BiP. To inhibit the addition of N-linked oligosaccharide to the

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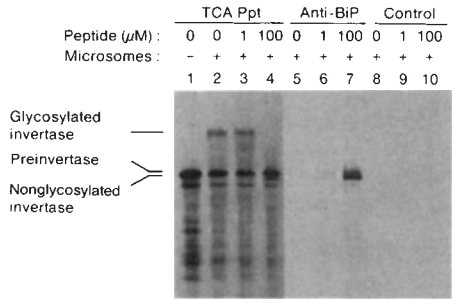

Fig. 1 Bip associates with incorrectly glycosylated invertase but not with native invertase. Composite fluorograph of an SDS polyacrylamide gel. Lanes 1-4, the trichloroacetic acid (TCA)-precipitable polypeptides synthesized *in vitro* from preinvertase mRNA under different reaction conditions: lane 1, products synthesized in the absence of dog pancreas microsomes; lanes 2-4, products synthesized in the presence of microsomes and the indicated concentrations of the tripeptide Ac-Asn-Tyr-Thr-NH_2; lanes 5-10, immune precipitations of aliquots of the same reactions, with a monoclonal antibody against BiP (lanes 5-7) or an unrelated control monoclonal antibody (lanes 8-10). Lanes 5-10 were exposed to film ~8 times longer than lanes 1-4.

Methods. Preinvertase mRNA was transcribed and capped *in vitro* as described[21] from the plasmid pS5 (provided by J. Ngsee and M. Smith). At time zero mRNA was added to wheat germ extract-dog pancreas microsome translation-translocation reactions (refs 22, 23) containing the tripeptide Ac-Asn-Tyr-Thr-NH_2 at the indicated concentrations. After 1 h at 26 °C the reactions were terminated by adding apyrase for 2 min at 37 °C (100 units ml^{-1} final concentration) to deplete ATP. Samples were then placed on ice and microsomes were lysed with Nikkol (0.1% final concentration). Aliquots of the reaction were withdrawn and precipitated with TCA (15% final concentration) or monoclonal antibodies. Samples for immune precipitation were incubated with monoclonal culture supernatants directed against either BiP[2] or an unrelated control antigen at 4 °C overnight. Protein A Sepharose was then added for 45 min at room temperature and the resultant immune precipitates were washed with 0.1% Nikkol in HEPES buffered saline. All precipitates were solubilized with SDS and analysed by electrophoresis on 10-18% gradient SDS polyacrylamide gels. Samples were visualized by fluorography with sodium salicylate. The tripeptide was synthesized at the Biomolecular Resource Center at USCF. HPLC analysis showed a purity of over 99%. Protease protection experiments (not shown) indicated that the tripeptide did not interfere with the translocation process.

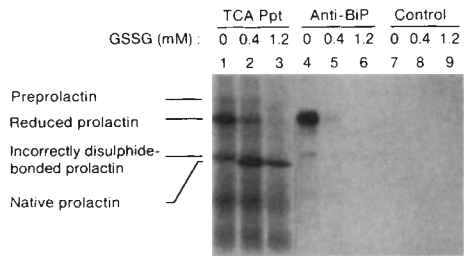

Fig. 2 BiP associates with reduced or incorrectly disulphide-bonded prolactin but not with native prolactin. Composite fluorograph of a non-reducing SDS polyacrylamide gel: lanes 1-3, TCA precipitable polypeptides synthesized *in vitro* from preprolactin mRNA in the presence of microsomes and the indicated concentrations of oxidized glutathione; lanes 4-9, immune precipitations of aliquots of the same reactions, with a monoclonal antibody against BiP (lanes 4-6) or an unrelated control monoclonal antibody (lanes 7-9). Lanes 4-9 were exposed to film ~7.5 times longer than lanes 1-3.

Methods. Preprolactin mRNA was transcribed and capped *in vitro* as described[21]. Translation-translocation reactions (refs 22, 23) containing oxidized glutathione (GSSG) in place of the tripeptide at the indicated concentrations are described in Fig. 2. After termination, samples were placed on ice and microsomes were lysed with Nikkol (0.1% final concentration) and alkylated with iodoacetamide (20 mM final concentration). Aliquots of the reaction were withdrawn and precipitated with TCA or monoclonal antibodies as described in Fig. 1. All precipitates were solubilized with SDS and analysed by electrophoresis on 10-18% gradient SDS polyacrylamide gels under non-reducing conditions, similar to the procedures of Kaderbhai and Austen[14]. Under these conditions, native prolactin has the mobility indicated. It runs with reduced prolactin on a reducing gel (not shown). 'Reduced prolactin' migrates identically on both reducing and non-reducing gels. The band marked "Incorrectly disulphide-bonded prolactin" migrates as reduced prolactin on a reducing gel but is slower than native prolactin on a non-reducing gel. Samples were visualized by fluorography with sodium salicylate.

protein we used the tripeptide Ac-Asn-Tyr-Thr-NH_2 which competes with nascent polypeptide chains as an acceptor for carbohydrate addition[12] (Fig. 1, compare lane 2 with lane 4). Signal peptide cleavage occurs normally, allowing translocated (non-glycosylated) chains to be distinguished from peptides which have not been translocated into the microsomes. Immune precipitation of BiP using a monoclonal antibody[2] reveals an association of BiP with a proportion (23%) of the incorrectly glycosylated invertase chains (Fig. 1, lane 7). No interaction of BiP with correctly glycosylated invertase can be detected under these conditions (Fig. 1, lanes 5 and 6). We conclude that BiP can specifically recognize and bind tightly to aberrantly glycosylated peptides *in vitro*. In Fig. 1 the incorrectly glycosylated invertase appears as a doublet (lane 7). Although the upper band comigrates with preinvertase, it cannot be the untranslocated precursor as association with BiP only occurs when glycosylation is blocked. Instead, the two bands probably represent unglycosylated and partially glycosylated invertase.

We next asked whether changes in protein structure other than altered glycosylation could lead to complex formation with BiP. To examine whether BiP can recognize changes in protein folding in the absence of altered amino acid or carbohydrate composition we used bovine prolactin as a model protein. Prolactin is a single polypeptide chain of 199 amino acids that contains three intra-chain disulphide bonds and is not glycosylated. The *in vitro* protein translation system normally contains dithiothreitol in sufficient concentration to inhibit disulphide-bond formation[13] but they can be made to form *in vitro* by altering the redox potential using oxidized glutathione[13,14]. The extent of disulphide bond formation can be monitored on non-reducing SDS-PAGE, where disulphide-bonded prolactin migrates faster than reduced prolactin, presumably due to its more compact configuration[14]. Figure 2 shows the results of an experiment in which prolactin has been synthesized with varying degrees of disulphide bonding. Lane 1 shows the products synthesized in the absence of oxidized glutathione. Most of the prolactin migrates with the mobility of reduced prolactin although 22% migrates more rapidly (but not with the mobility of native prolactin) showing that it contains some disulphide bonds. In lanes 2 and 3, where oxidized glutathione is added to the reaction, a new species with the mobility of native prolactin appears. Immune precipitation of BiP reveals the specific association of BiP with a fraction (7%) of the reduced prolactin chains (lane 4). In addition a smaller amount (about 1%) of the prolactin that is incorrectly disulphide-bonded co-precipitates with BiP. No correctly disulphide-bonded prolactin co-precipitates with BiP. This experiment clearly shows that BiP can discriminate between nonglycosylated polypeptides of identical amino acid composition and can form a tight association with polypeptides which are aberrant only in folding and disulphide bonding.

There is evidence that proteins fold, form disulphide bonds, and even oligomerize, before synthesis is complete[15,16]. If BiP were to facilitate one of these steps, an interaction of BiP with nascent polypeptide chains might be expected. To detect association of BiP with nascent chains, the rate of synthesis was slowed

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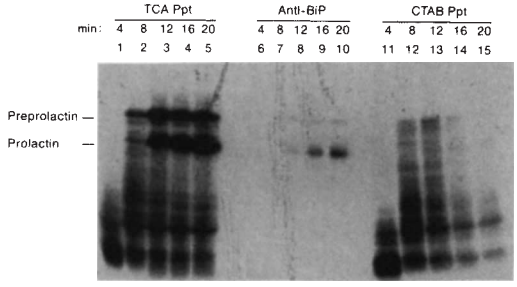

Fig. 3 BiP associates with mature prolactin but not with nascent chains. Fluorograph of a reducing SDS polyacrylamide gel: lanes 1-5, TCA precipitations of polypeptides synthesized at different times *in vitro* in a synchronized translation using preprolactin mRNA in the presence of microsomes; lanes 6-10, immune precipitations of aliquots from the same time points with a monoclonal antibody against BiP; lanes 11-15, aliquots that have been precipitated with CTAB which precipitates nascent chains covalently bound to tRNA[17].

Methods. At time zero preprolactin mRNA was added to a wheat germ extract/dog pancreas microsome translation-translocation system at 22 °C. After 4 min 7-methyl guanosine (5 mM final concentration) and edeine (5 μM final concentration) were added to block further initiation of peptide synthesis. At the indicated times aliquots of the reaction were withdrawn and treated with glycerol kinase (250 μg ml^{-1} final concentration) and glycerol (5% final concentration) for 1 min at 22 °C to deplete ATP and samples were then placed on ice. Each time point was split into three fractions for precipitation with TCA, CTAB[24], or with monoclonal antibody to BiP (as in Fig. 1). Precipitates with CTAB and TCA were treated with 1 M Tris base and boiled in SDS to remove the tRNA. All precipitates were solubilized with SDS, reduced with 2-mercaptoethanol, and analysed by SDS-PAGE followed by fluorography as described in Fig. 1.

by performing the reaction at 22 °C instead of 26 °C, and the *in vitro* translation system was synchronized by adding preprolactin mRNA at time zero. Four minutes later, the initiation of new peptide chains was inhibited with edeine and 7-methyl guanosine. Those chains already initiated continued to elongate. At various times during the course of the reaction aliquots were withdrawn and analysed in three ways: acid precipitation of the lysate revealed the total products synthesized; precipitation with the detergent hexadecyltrimethyl ammonium bromide (CTAB) allowed the visualization of nascent chains still covalently bound to transfer RNA[17]; and immune precipitation of BiP revealed products that were tightly bound to BiP (Fig. 3). As the redox conditions of this reaction prevent correct disulphide-bond formation, BiP is found tightly associated with a fraction of the full-length prolactin chains (lanes 8, 9 and 10) consistent with the data from non-reducing gels (Fig. 2). None of the nascent chains observed in lanes 11-15 co-precipitated with BiP (lanes 6-10) however. A tight association of BiP with nascent prolactin chains is not detectable even after considerably longer exposure of the gel (not shown).

Not all the preprolactin synthesized *in vitro* is translocated across the membrane and processed by signal peptidase. Densitometry of the gels (Fig. 3) shows that the ratio of prolactin to preprolactin is 3 to 1. Preprolactin is also found in the immunoprecipitates generated with anti-BiP antibodies. This is unlikely, however, to represent specific association of BiP with the extraluminal preprolactin after microsome lysis as in control experiments (not shown) preprolactin (but not prolactin) shows strong non-specific adsorption to the immunoadsorbent even in the absence of anti-BiP antibodies. Incomplete preprolactin chains, whether translocated or not, cannot be detected in the immunoprecipitate, with or without anti-BiP antibodies.

In interpreting the above results, it is important to acknowledge that the technique of immune precipitation used would only detect BiP complexes of high affinity and that BiP might also enter into rapidly dissociable interactions with proteins that we would not have detected. The conclusion that BiP specifically binds to aberrant proteins, and not to native proteins or to nascent polypeptides, is therefore only true for the high affinity interactions described in this study. The *in vivo* experiments described earlier[1-4,7], have the same limitations.

If BiP played an essential role in folding or disulphide bond formation of normal proteins, it should bind more extensively to nascent than to mature chains. Because a high affinity interaction with BiP was not observed for nascent prolactin chains, we conclude that it cannot be a necessary step in the folding of all proteins as they enter the ER. We have shown that BiP can bind tightly to aberrantly folded or glycosylated proteins *in vitro* so why are nascent prolactin chains not recognized as aberrant? As incompletely synthesized proteins would not have a native conformation, conformation alone cannot be the feature that BiP recognizes on aberrant proteins. It has been suggested that BiP recognizes surface sites of high hydrophobicity[3,4]. To reconcile such a model with the data presented here, the site which BiP recognizes on misfolded prolactin chains must be inaccessible on nascent chains. The peptide bands seen in Fig. 3, lanes 11-15, which shows nascent chains, are generated by a poorly understood 'pausing' mechanism. At least 70% of the two predominant bands, 87 and 120 amino acids long, are undergoing translocation (Siegel and P.W., manuscript in preparation) so that 50 amino acids which span the ribosome and the ER membrane[18] would be inaccessible for BiP binding. The N-terminal 70 amino acids that are accessible, however, clearly do not associate with BiP. If BiP were to bind to a site within the last 50 amino acids of prolactin, this site would be unavailable until after synthesis and translocation were completed. Another possibility is that BiP binds only to aggregates of proteins in the ER. This possibility has been previously suggested based on similarity between BiP and other members of the HSP-70 family[4,19] which are proposed to dissolve protein aggregates (such as those which might form during heat shock). Incorrectly glycosylated or folded proteins might be more likely to aggregate than native molecules[20], whereas nascent chains might be prevented from aggregating by their association with the translocation machinery in the ER membrane.

In this *in vitro* system only a fraction of the polypeptide chains that have incorrect disulphide bonds or are not glycosylated are recovered in the BiP immune precipitates. The low recovery could be due to inefficient association with BiP in our *in vitro* translation-translocation system, or dissociation of BiP complexes during the immune precipitation procedures. Alternatively, BiP might recognize only a subset of the aberrant molecules. The protein aggregation model[4,19], for example, might predict that only aggregates of aberrant proteins would be bound by BiP.

We have used an *in vitro* system to study the interaction of BiP with newly synthesized proteins so that we could manipulate the conditions of protein synthesis, glycosylation and folding. In addition it eliminates other variables such as changing levels of BiP due to induction and the transport of proteins out of the ER. Our data show that BiP can distinguish between native and aberrant proteins and can form tight complexes with misfolded or aberrantly glycosylated polypeptides *in vitro*. What is the function of such associations *in vivo*? Several observations suggest that BiP is not merely an intracellular garbage collector. Bole *et al.*[2] have presented evidence that immunoglobulin heavy chains and incompletely assembled oligomers associate transiently with BiP during normal immunoglobulin assembly. It has also been reported that isolated BiP-heavy-chain complexes can be disrupted *in vitro* with ATP[4]. Consistent with this observation, we found it necessary to deplete the ATP in the translation reaction before microsome lysis in order to isolate BiP-protein complexes from our *in vitro* system; if microsomes were lysed without depleting ATP, no peptides co-precipitated with BiP (not shown). It is hard to reconcile an energy-dependent

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dissociation of BiP with a model in which BiP's sole function is to bind to aberrant proteins. BiP's characteristic association with aberrant proteins is more likely to be due to a futile attempt to correct their aberration than a preference for their company. The availability of an *in vitro* system should help to elucidate the role of this highly conserved protein.

We thank Dr David Bole for providing monoclonal antibody against BiP, and members of the Kelly lab for critical reading of the manuscript. This work was supported by NIH grants to P.W. and R.B.K.

Received 25 January; accepted 30 March 1988.

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Intraorganellar calcium and *p*H control proinsulin cleavage in the pancreatic β cell via two distinct site-specific endopeptidases

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Insulin is produced from an inactive precursor, proinsulin, through initial endoproteolytic cleavage at sites marked by pairs of basic amino-acid residues[1,2]. We report here that lysates of insulin secretory granules contain two distinct Ca-dependent acidic endoproteases; one (type I) cleaving exclusively on the C-terminal side of Arg 31.Arg 32 (B-chain/C-peptide junction), the other (type II) preferentially on the C-terminal side of Lys 64.Arg 65 of proinsulin (C-peptide/A-chain junction). The Ca and *p*H requirements of these proteinases suggested that the type-II proteinase would be active in the Golgi apparatus and the secretory granule, whereas type-I activity would be compatible only with the intragranular environment. Kinetic analyses of (pro)insulin conversion intermediates in [^{35}S]methionine-pulsed rat islets support this supposition. Our results suggest a simple mechanism whereby different dibasic sites can be cleaved in different cellular compartments. In conjunction with the regulation of the ionic composition of such compartments and the operation of post-Golgi segregation, our results also suggest how proteolytic conversion of diverse proproteins destined for different cellular sites can occur differentially and in a regulated manner.

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Previous studies have shown that the processing of ^{125}I-labelled human proinsulin[3] and of biosynthetically labelled chromogranin A (ref. 4) by lysed subcellular fractions of a transplantable insulinoma is initiated by a novel Ca^{2+}-dependent endopeptidase activity which is localized principally to the secretory granule compartment. The activity in purified insulinoma granules was solubilized by sonication in media containing 0.5% Triton X-100 and subjected to chromatography on DEAE-cellulose. Fractions were collected directly onto 'spun' columns of G-25 Sephadex equilibrated with 50 mM acetate buffer *p*H 5.5 to avoid inactivation of these enzymes by anions contained in the eluting buffer[4]. Two distinct endopeptidase activities were observed (Fig. 1). The first (type-I activity) which eluted with the column void converted [^{125}I]proinsulin to 32,33-split proinsulin (Fig. 2). The second (type-II activity) which eluted between 0.25–0.3 M NaCl converted the substrate principally to 65,66-split proinsulin and, to lesser extent, 32,33-split proinsulin and diarginylinsulin (insulin with the B-chain extended by Arg 31.Arg 32). The relative ratio of the latter three products produced by different fractions over the peak of type II activity (Fig. 1) remained essentially constant, a finding consistent with the presence of a single enzyme which cleaves both dibasic sequences in proinsulin but the A-chain/C-peptide junction preferentially.

Identification of the split proinsulin intermediates was on the basis of their co-elution on high pressure liquid chromatography (HPLC) with standards, their conversion by carboxypeptidase B or H via C-terminally extended monobasic forms to des 31,32- and des 64,65-proinsulin respectively, and their conversion to insulin and diarginyl insulin respectively by tryptic proteolysis (30 min at 30 °C with 10 ng trypsin in 100 μl 50 mM Tris formate *p*H 7.5; results not shown). These assignments were confirmed by alkaline urea gel electrophoresis and acid urea-gel electrophoresis as appropriate. The two endopeptidase activities when recombined, generated diarginylinsulin in addition to the split proinsulins as expected. This was a similar spectrum of intermediates as generated by limited trypsinolysis of [^{125}I] proinsulin and also by lysed secretory granule preparations in which the endogenous carboxypeptidase H was inhibited by adding 1 μM guanidinoethanemercaptosuccinic acid[3]. The combination of the two endopeptidase activities with carboxypeptidase H was required to produce insulin. The yield of insulin under suitable conditions reached 80% and there was no evidence of further degradation of the insulin molecule upon extended incubation (16–24 h).

The type I and II endopeptidase activities prepared as in Fig. 1 but in the absence of protease inhibitors were shown to be insensitive to group-specific inhibitors of serine (1 mM diisopropylfluorophosphate), thiol (1 mM iodoacetamide) and aspartyl proteinases (1 mM pepstatin A). Both were inhibited by the chelating agents EDTA and 1,2 cyclohexanediaminetetraacetic acid (CDTA) but not by the heavy metal chelator 1,10 phenanthroline. Following removal of the chelator by gel filtration, activity was completely restored by addition of 5 mM Ca^{2+}. But whereas the Lys 64.Arg 65-directed type II activity was reactivated by micromolar Ca^{2+} concentrations ($K_{0.5}$ = 100 μM), the Arg 31.Arg 32-directed type I activity required millimolar Ca^{2+} ($K_{0.5}$ = 2.5 mM) (Fig. 3). The *p*H optimum of both activities was 5.5; however, the type II activity was distributed over a broader *p*H range (Fig. 3). This meant that at *p*H 7.5 type II activity was around 30–40% of that at *p*H 5.5, whereas the type I activity was negligible. These data suggested that both activities would be maximal in the ionic environment of the secretory granule where the *p*H approximates 5.5 (ref. 5) and the ionized Ca^{2+} concentration is 1–10 mM (ref. 6). In the Golgi apparatus and the condensing vacuole, through which these enzymes presumably pass en route for the granule, only the type II activity would be significant as the *p*H here is closer to neutrality[7] and Ca^{2+} is present at a lower concentration[8].

These findings raised the question of whether cleavage of the

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