Des-(27–31)C-Peptide

A NOVEL SECRETORY PRODUCT OF THE RAT PANCREATIC BETA CELL PRODUCED BY TRUNCATION OF PROINSULIN CONNECTING PEPTIDE IN SECRETORY GRANULES*

(Received for publication, January 11, 1996, and in revised form, May 21, 1996)

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Proinsulin conversion occurs within immature secretory granules (1, 2) resulting in the production of equimolar amounts of insulin and C-peptide (3). These molecules are stored in mature secretory granules until they are released from the beta cell. It has thus been widely assumed that insulin and C-peptide must always be released in equimolar amounts (4–6). However, this assumption is based upon the tenet that insulin and C-peptide are equally stable within beta cell secretory granules and thus their relative proportion does not change with time. Such is not always the case.

First, C-peptide can be subject to endoproteolytic cleavage. If such cleavage is restricted to one or only few sites in the molecule, it leads to the production of discrete truncated forms of the peptide (7, 8). If such truncation occurs in the beta cell secretory granule (rather than lysosomes), it will lead to the secretion of these truncated forms. More extensive proteolysis will result in the degradation of C-peptide without any detectable truncated forms, and this appears to be the case in transformed rat beta cells (INS cells), but not in isolated adult rat islets (9). Both limited and extensive proteolysis will elevate the ratio of insulin to intact (non-truncated) C-peptide to values greater than one. Second, a novel post-granular, constitutive-like secretory pathway has been proposed in which vesicles bud from granules carrying C-peptide and not insulin leading to selective release of C-peptide from the beta cell (10). When this occurs, the secretory granules will be left with an insulin to C-peptide ratio greater than unity.

Our previous data suggested the presence of a discrete truncated form of C-peptide in secretory granules of INS cells, as well as more extensive degradation (9). In the present work, we identified this truncated peptide by sequence analysis and mass spectrometry as a novel form of C-peptide lacking the 5 C-terminal residues (des-(27–31)C-peptide). We further show that this truncated C-peptide is a major secretory product of both neonatal and adult rat beta cells and as such not a peculiarity of insulinoma cells. Both truncation and complete degradation appear to contribute to non-equimolar secretion of insulin and C-peptide from beta cells of newborn rats, whereas only truncation is apparent in granules of beta cells from adult rats.

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture—Monolayer cultures were prepared from the pancreases of neonatal (3- to 5-day-old) Sprague-Dawley rats as described previously (11). Studies were performed following 3 days of growth in culture medium consisting of 45% NCTC 133, 45% medium 199 (v/v; Life Technologies, Inc., Grand Island NY) and 10% fetal calf serum supplemented with 16.7 μM glucose and 50 μg/ml gentamicin.

Adult rat islets were isolated from male Sprague-Dawley rats weighing 200–220 g by collagenase digestion as described previously (12). Prior to experiments the islets were cultured overnight in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 8.3 mM glucose.
Note that in separate experiments we have confirmed that neither the choice of Dulbecco's modified Eagle's medium for adult islets versus the mixture of 45% NCTC 135, 45% medium 199 used for the newborn islet monolayers nor the glucose concentration (8.3 versus 16.7 mM) influence the stability of C-peptide within adult islet cells. The choice of Dulbecco's modified Eagle's medium with 8.3 mM glucose for the adult islets was based upon previous studies demonstrating that under these conditions islets remain the most viable with well maintained secretory responses. Longer incubation periods resulted in a decreased balance between secretion and intracellular degradation (erophagy) (13).

**Expression of Human Proinsulin in INS Cells Using Recombinant Adenovirus**—Recombinant adenovirus for expression of human proinsulin was prepared by homologous recombination of the plasmid pRM17 (14), which consists of a modified Ad5 genome, and pACCMV-hins, which encodes the poly(A) adenosine 3' to 5' polymerase (15) by inserting human proinsulin cDNA into the SmaI site of the polylinker of pACCMV, pLPa (15). To this end, semi-confluent 293 cells were co-transfected with the two plasmids using a modified calcium phosphate precipitation method (16). The 293 cells were seen to lyse some 2–3 weeks post-transfection and clonal recombinant virus was then prepared by reinfestation of 293 cells followed by overlay with 0.7% agar. Plaques were visible 7–10 days later and were taken to correspond to viral clones. INS cells were maintained in tissue culture as described in detail previously (17), and were seeded in 35-mm diameter Petri dishes at a density of 10^6 cells/dish. Four days after seeding the dishes, the cells were infected with recombinant adenovirus for 1 h at a multiplicity of infection of 13. After washing, the cells were kept in culture for 24 h before being used for the pulse-chase experiments.

**Pulse-Chase Methodology**—Following culture, neonatal rat beta cells were washed 3 times in 1.0 mL of prewarmed Krebs-Ringer bicarbonate buffer (KRB) containing 0.1% bovine serum albumin, 20 mM Heps, 1.67 mM glucose, and no calcium. The cells were preincubated in 1.0 mL of this medium for 15 min at 37°C and then pulse labeled for 10 min at 37°C in 0.30 mL of KRB containing 400 μCi/mL [3H]leucine (Amersham Corp.) and 16.7 mM glucose. The labeled cells were washed 3 times with 1.0 mL of ice-cold KRB containing 1.67 glucose and 1.0 mL unlabelled leucine, then chased for 2 or 10 h at 37°C in medium composed of 45% NCTC 135, 45% medium 199 with 20 mM Heps, 5.5 mM sodium bicarbonate, 10% fetal calf serum, and 8.3 mM glucose. Human C-peptide (10 μg/mL; Peninsula Laboratories, Belmont, CA) and human insulin (100 μg/mL; Eli Lilly and Co., Indianapolis, IN) were also added to prevent degradation of labeled C-peptide and insulin in the media. In some wells, NH4Cl was added after 2 h of chase to obtain a final acidification in the medium of 25 mM. Following the chase period, the medium was harvested and the cells were harvested by 3× 0.5 mL of 1 M acetic acid followed by 5 ml min centrifugation at 10,000 rpm to remove insoluble material. Chase media and cell extracts were stored at -20°C prior to analysis.

Adult rat islets were pulse-labeled and chased using a similar protocol with the following modifications. The 10-min pulse label was in a volume of 0.2 mL of KRB containing 500 μCi/mL [3H]leucine (ARC, St. Louis, MO) and the 2- or 10-h chase was in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 8.3 mM glucose.

INS cells were taken 24 h after infection with recombinant adenovirus. Non-infected cells were used as controls. The cells (2× 10^6 cells in 35-mm diameter Petri dishes) were pulse-labeled (20 min, 50 μCi of [3H]leucine in 0.8 mL of KRB, 16.7 mM glucose) and then chased for 2 h under basal conditions (KRB, 1.67 mM glucose).

**Stimulation of Regulated Granule Exocytosis**—Following chase periods of either 2 or 10 h, exocytosis of beta cell secretory granules was stimulated to allow assessment of labeled peptides in secretory granules. To this end, at the end of the 2- or 10-h chase, cells were washed 3 times in ice-cold KRB containing 1.67 mM glucose, and thereafter 500 μl of a pre-warmed stimulation buffer containing a mixture of beta cell secretagogues was added (KRB supplemented with 16.7 mM glucose, 0.1 mM isobutylmethylxanthine (Sigma, St. Louis, MO), 5 mM carbachol (Sigma), and 10 mM arginine). After 1 h of incubation at 37°C, the stimulation medium was collected and the cells extracted in acid as above. All samples were stored at -20°C prior to analysis.

In order to discharge INS cell granule contents, following 2 h of chase INS cells were stimulated for 15 min with KRB containing 16.7 mM glucose, 5 mM forskolin, 0.1 mM phenol, 12-myristate 13-acetate, 0.1 mM Glutamine (9). Isolation of Nuclear DNA—The technique for surgical isolation and vascular perfusion of the pancreas has been previously described (18). Male Wistar rats weighing 300–350 g were used. The perfusate was a Krebs-bicarbonate buffer containing 0.2% bovine serum albumin, 3% dextran (Clinical grade; Sigma) and either 4.4 or 16.7 mM glucose. It was gassed with water-saturated 95% O2, 5% CO2 to maintain a physiological pH, heated to 37°C and pumped through the pancreas via the abdominal aorta at a flow rate of 3 mL/min. Glucose was administered as follows: min 0–20, 4.4 mM; min 20–55, 16.7 mM; min 55–80, 4.4 mM. Five-minute fractions (15 mL) of portal venous effluent were collected in chilled plastic vials containing 500 μl of acetic acid (10%) to inhibit protease activity.

Note that in separate experiments we have confirmed that neither the choice of Dulbecco's modified Eagle's medium for adult islets versus the mixture of 45% NCTC 135, 45% medium 199 used for the newborn islet monolayers nor the glucose concentration (8.3 versus 16.7 mM) influence the stability of C-peptide within adult islet cells. The choice of Dulbecco's modified Eagle's medium with 8.3 mM glucose for the adult islets was based upon previous studies demonstrating that under these conditions islets remain the most viable with well maintained secretory responses. Longer incubation periods resulted in a decreased balance between secretion and intracellular degradation (erophagy) (13).
Secretion of Truncated C-Peptide from Rat Beta Cells

Characterization of Rat Truncated C-peptide—HPLC analysis of rat cell extracts and media allowed for the separation of rat insulin I and II as well as rat C-peptide I and II. HPLC profiles of media from neonatal rat beta cells revealed the presence of a pair of radioactive peaks which eluted approximately 20 min earlier than the two intact C-peptides (Fig. 1). Preliminary studies on these peptides had suggested that they were truncated C-peptide molecules (9) but they had yet to be characterized definitively.

In order to identify the precise site of truncation of rat C-peptide, four approaches were used: amino acid composition, partial N-terminal sequencing, C-terminal amino acid analysis, and mass spectrometry. The amino acid composition of the truncated form of both C-peptide I and II suggested that the last five residues of C-peptide I had been lost (data not shown). This hypothesis was reinforced by sequencing the N-terminal region of the truncated molecules which showed that this region was unaltered compared to intact C-peptide I or II (sequence obtained: EVEDPQVF for truncated C-peptide I and EVEDPQVIA for truncated C-peptide II). The C-terminal region of the truncated C-peptide I was analyzed by digestion with carboxypeptidase. The results were again in keeping with the loss of the last five residues (Leu, 190 pmol; Ala, 110 pmol; Thr, 52 pmol; Gln, 44.5 pmol; Asp, 41.7 pmol; Gly, 60.5 pmol). Definitive identification of the truncated rat C-peptides was based upon their molecular mass determined by mass spectrometry. The intact C-peptides were included in this study as a control. As detailed in Table I, the predicted and observed masses for the intact C-peptides were close to identical. The observed mass for the truncated forms of both C-peptides confirms unequivocally that the truncation results in the loss of five residues from the C terminus leading to the generation of des-(27–31)C-peptide I and II (Fig. 2).

Neonatal Rat Islet Cell Monolayers—As discussed under “Experimental Procedures,” only results for insulin I and C-peptide I (and its truncated form) are presented. Radioactive insulin, C-peptide, and des-(27–31)C-peptide were quantified by HPLC in cell extracts after 2 and 10 h of chase, and in the medium following 1-h stimulation of the cells with a secretory mixture containing isobutylmethylxanthine, carbachol, arginine, and glucose. Such stimulation resulted in the release of greater than 50% of cellular labeled insulin after both 2 and 10 h of chase. Products released during the 1-h stimulatory period are taken to reflect the contents of secretory granules at the two times of chase.

Significant amounts of label were recovered in the form of des-(27–31)C-peptide in both cells and granules. After 2 h of chase, 11.9 ± 0.2% of granular labeled C-peptide was truncated.
The 1-h stimulated medium), was 12.2.

Since selective loss of C-peptide from granules might occur by constitutive-like secretion of vesicles enriched in C-peptide relative to insulin (10), we assessed the activity of this pathway in these cells by measuring I/(CP + tCP) in the 2- and 10-h chase medium, prior to the 1-h stimulation period. Release of C-peptide via this pathway is predicted to result in an I/CP ratio less than one in the medium (10). Surprisingly, I/(CP + tCP) in the pre-stimulation chase medium was close to 1 at both time points (2 h, 0.96 ± 0.04; 10 h, 1.02 ± 0.02). Since no extracellular degradation of either insulin or C-peptide was observed (see “Experimental Procedures”) and I/(CP + tCP) in secretory granules was greater than 1 (see above), the equimolar release of labeled insulin and C-peptide observed during the pre-stimulation chase period must be due to the net contributions of both constitutive-like secretion (I/(CP + tCP) < 1) and granular secretion (I/(CP + tCP) > 1). Thus, C-peptide loss via constitutive-like secretion occurs in neonatal rat beta cells and contributes to the I/(CP + tCP) > 1 present in secretory granules. However, it can be calculated that this pathway only accounted for 1.7 and 9.1% of the C-peptide selectively lost from granules during the 2- and 10-h chase periods, respectively. Thus, the majority of C-peptide selectively lost from granules as reflected in the elevated I/(CP + tCP) in the 1-h stimulated media was due to degradation rather than constitutive-like secretion.

Insulin secretory granules are known to be acidic (1, 24). We reasoned that the selective loss of C-peptide from granules and/or truncation may be dependent on this acidic environment. Therefore, we examined the impact of NH4Cl on C-peptide, since this agent has been shown to effectively restore the pH of the intragranular milieu to neutrality (24) and we have shown it to inhibit loss of C-peptide from INS cell secretory granules.5 However, since proinsulin to insulin conversion is dependent on granular acidification (24–26), we added NH4Cl only after the first 2 h of chase, in order to allow extensive conversion of newly synthesized (labeled) proinsulin to have occurred. Addition of NH4Cl for the last eight of the 10 h of chase was followed by 1 h stimulation with the secretory mixture (without NH4Cl). I/(CP + tCP) in the 1-h stimulated medium of NH4Cl-treated cells was significantly lower than that of cells that were chased for 10 h in the absence of NH4Cl (1.14 ± 0.04 versus 1.50 ± 0.03; p < 0.001; Fig. 4, lower panel, hatched bars). In fact, I/(CP + tCP) in the NH4Cl-treated cells

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was identical to that observed following only 2 h of chase. Thus, since NH4Cl had only been present in the chase medium as from the 2-h time point, it had totally inhibited the selective loss of C-peptide from secretory granules during the last eight h of the 10-h chase. Although I/(CP loss of C-peptide from secretory granules during the last eight of the 10-h chase. NH4Cl than without (1.58 

**Expression of Human Proinsulin in INS Cells**—The comparison of the sequence of rat C-peptides I and II with that of human C-peptide (Fig. 2) revealed conservation at the site cleaved in the generation of des-(27–31)/C-peptide. This led us to suspect that human C-peptide may also be susceptible to truncation in the same way as the rat peptides. In order to monitor the truncation of rat and human C-peptide in the same cellular setting we took advantage of recombinant adenovirus to express human proinsulin at high levels in transformed INS cells. We have shown previously that truncation of rat C-peptides is an active process in these cells (9). Following a pulse label and a 2-h chase, the INS cells were stimulated for 15 min and products released during this period analyzed by HPLC. The infected cells released two radioactive products which co-

**Fig. 5. Ratio of labeled insulin to C-peptide in adult rat islet beta cells.** Isolated islets were pulse-chased and analyzed as described in the legend to Fig. 3. In addition, some cells were extracted at the end of the 2- and 10-h chase periods. The ratio of insulin to intact C-peptide is shown in the open bars, and that of insulin to the sum of intact C-peptide and des-(27–31)/C-peptide in the hatched bars. Upper panel, cell extracts; lower panel, 1-h stimulated medium (granules; see legend to Fig. 3). Data are presented as mean ± S.E., n = 3.

**Fig. 6. Release of truncated C-peptide II (des-(27–31)/C-peptide II) from the perfused adult rat pancreas.** Pooled perfusates collected during the periods indicated under each set of bars (with glucose concentration in the parentheses) was analyzed by HPLC and intact and des27–31/C-peptide II measured in fractions by radioimmunoassay. Panel A, incremental increase (above pre-stimulus) in rate of release of des(27–31)/C-peptide II; Panel B, percent of total C-peptide II (intact plus des<27–31)/C-peptide II) comprised of des-(27–31)/C-peptide II. Data are presented as mean ± S.E., n = 3.

**Panel A**

![Graph showing ratio of labeled insulin to C-peptide in adult rat islet beta cells.](image)

**Panel B**

![Graph showing release of truncated C-peptide II (des-(27–31)/C-peptide II) from the perfused adult rat pancreas.](image)
is lost from secretory granules. Such selective loss results in cells (INS cells), we have found that C-peptide but not insulin shown that this is not always the case. In transformed beta cations of the relative proportion of insulin and C-peptide secretory granules (in contrast tocreted from the beta cell.

Such is not the case for C-peptide, which is located in the halo granules and the relative stability of insulin within multicellular products if they are not released (13, 27). Such degradation arises by crinophagy (28). This process, common to all secretory products, involves fusion of secretory granules with lysosomes resulting in the formation of multigranular bodies (29), the classic degradative compartment for granular products. Once insulin and C-peptide have been targeted to this degradative compartment, they are no longer subject to regulated secretion. In contrast to the beta cell, where insulin is synthesized and stored in secretory granules, and the relative stability of insulin within multinuclear bodies has been attributed to its crystal state (28, 30). Such is not the case for C-peptide, which is located in the halo of the secretory granule (28, 31), is soluble, and is thus degraded very rapidly once introduced into the lysosome (28, 30). The result of this notable difference in stability of these two molecules formed as a result of proinsulin processing is an elevated ratio of insulin to C-peptide in extracts of beta cells (19). Measuring such ratios in extracts of whole cells does not, however, provide an accurate indication of the situation prevailing in secretory granules and thus cannot provide an indication of the relative proportion of insulin and C-peptide secreted from the beta cell.

It is commonly assumed that both insulin and C-peptide are equally stable within beta cell secretory granules (in contrast to multigranular bodies) and that they will thus be secreted in equimolar amounts. However, recent work from our group has shown that this is not always the case. In transformed beta cells (INS cells), we have found that C-peptide but not insulin is lost from secretory granules (9). Such selective loss results in the disproportionate release of insulin relative to C-peptide. In the present study, we demonstrate that the selective loss of granular C-peptide is not unique to transformed cells but that this process is also operative in primary neonatal (but not adult) rat beta cells. In addition to the selective loss of granular C-peptide, we have determined that limited proteolysis of C-peptide also occurs in beta cell secretory granules of both neonatal and adult rats, resulting in the production and secretion of a truncated form of C-peptide, des-(27–31)C-peptide.

Truncation of C-peptide was first shown in 1973 to occur within beta cells (8) but has been largely ignored since that time. In the initial description of this occurrence in rat islets, it was suggested that cleavage of C-peptide arose prior to proinsulin conversion (8). The conversion process would then liberate a truncated form of C-peptide. This truncated form (C-peptide I–22 or des-(23–31)C-peptide to use currently accepted nomenclature) was lacking its nine C-terminal amino acids (8). The truncated C-peptide described in the present study differs both in its mode of generation and its composition. We have found that the proportion of this particular truncated form increases between 2 and 10 h of chase. Given that proinsulin conversion is complete within granules of newborn rat beta cells (27, 32), this increase thus suggests that the truncated form studied here is generated from free C-peptide as well as possibly from proinsulin. Furthermore, molecular characterization of the truncated forms of both C-peptide I and II show clearly the loss of just the last five, not nine, residues. Note, however, that in the present study we have not attempted to identify other truncated forms of C-peptide by HPLC and our data thus do not exclude the existence of the previously identified truncated form of C-peptide (8) or, indeed, yet smaller forms.

The detailed molecular characterization of truncated C-peptide I and II was performed on peptides from adult rat islets. It is assumed that the same truncation is also occurring in the newborn cells based upon the elution times from HPLC. Furthermore, based on the known amino acid sequences of rodent and human C-peptide, we predicted that this truncation event could occur in human C-peptide given that the same Leu26-Glu27 cleavage site is present in the human molecule (Fig. 2) and indeed in other higher species (33). To test this hypothesis, human proinsulin was expressed in transformed rat beta cells, and the human C-peptide stored in granules of these cells was indeed found to be truncated to roughly the same extent as its rat counterpart. It should be noted in this context that a cascade of different truncated C-peptides has recently been identified in extracts of a human insulinoma (7). As discussed above, the study of truncated peptides in tissue extracts does not provide any indication of the situation prevailing in secretory granules since the extracts will reflect the combined contents of granules and multigranular bodies. Thus, the finding of such truncated peptides in extracts does not necessarily imply that they are secreted by the beta cell. Moreover, it is also well established that truncation can arise as an artifact of extraction, and such has indeed been found for both dog (removal of the N-terminal octapeptide) (34) and rhinoceros (removal of the C-terminal octapeptide) (35) C-peptides. Our demonstration that des-(27–31)C-peptide is not only present in cell extracts, but is also secreted in a regulated manner from several different preparations (primary cells from newborn and adult rats, the perfused rat pancreas and transformed rat beta cells), tends to rule out that this truncation event is an artifact and rather suggests that it is a normal post-translational modification of C-peptide in the beta cell secretory granule. Two mechanisms have been proposed for the selective loss of C-peptide from granules (9). First, C-peptide could be degraded...
directly within secretory granules. Second, C-peptide could be selectively removed from secretory granules in microvesicles. Such vesicles could in turn have two fates. They could discharge their contents by exocytosis representing the post-granular constitutive secretory pathway suggested by some to be operative in secretory cells including the pancreatic beta cell (10, 36, 37). The data from this study suggest that the constitutive-like pathway makes a significant, albeit small contribution to the loss of C-peptide from secretory granules or being selectively transferred to lysosomes. Although neutralization with NH$_4$Cl indicates clearly that loss of granules or being selectively transferred to lysosomes. Alternatively, C-peptide is being degraded directly within secretory granules (9). A second fate of these microvesicles is that they could be targeted to lysosomes where the C-peptide would be degraded. Our data do not allow us to distinguish whether C-peptide is being degraded directly within secretory granules or being selectively transferred to lysosomes. Although neutralization with NH$_4$Cl indicates clearly that loss of C-peptide from within secretory granules is dependent upon compartmental acidification, this observation is compatible with either mechanism as both could be dependent on an acidic environment.

Truncation of C-peptide was much more evident in immature than in mature beta cells. However, even in the adult beta cell secretion of des(27–31)C-peptide was not trivial, comprising approximately 10% of total C-peptide immunoreactivity released from the perfused adult rat pancreas under both basal and glucose-stimulated conditions. Interestingly, unlike the process responsible for selective C-peptide loss from the secretory granule, the truncation process is not totally acid-dependent since it was not fully inhibited by addition of NH$_4$Cl. This suggests that these are separate processes although it is possible that truncation may be an early step in a more extensive degradative process. Alternatively, it is possible that the truncated form of C-peptide may represent a biologically important peptide or may be an intermediate in the formation of a yet smaller product of biological importance that we could not identify in our HPLC analysis.

The findings from the present study may have implications for clinical pathophysiology, both in terms of measurement and potential biological activity of C-peptide related molecules. If disease states in which beta cell function is perturbed (such as insulinoma or non-insulin-dependent diabetes mellitus) are associated with selective granular loss of C-peptide and consequently non-equimolar release of insulin and C-peptide, then use of C-peptide as a marker of functional status of the beta cell may underestimate true function. Furthermore, should truncated forms of human C-peptide escape detection by other analytical techniques, such as radioimmunoassay, then beta cell secretory function may similarly be underestimated. Finally, the identification and characterization of a novel truncated form of C-peptide, des(27–31)C-peptide, also raises interesting questions regarding the potential biological activity of C-peptide. Presently, a definite biological role of intact C-peptide has not been demonstrated (see Ref. 38, for review). If des(27–31)C-peptide is indeed released in humans, it may serve a biological function. It appears less likely that the C-terminal pentapeptide liberated by the truncation would have biological activity since this region of C-peptide is not well conserved (Fig. 2 and Ref. 33). The possibility that des(27–31)C-peptide is biologically active has not been considered in studies in which intact C-peptide has been administered in search of its biological function. Indeed, our data raise the possibility that intact C-peptide may itself not be a bioactive peptide but merely the biosynthetic precursor of such a peptide.

Acknowledgments—We are indebted to Isabelle Antoni, Jeanette Teague, Hong Nguyen, Sharon Olwig, and Pierre-Olivier Regnery for expert technical assistance and thank Dr. Daniel Porte, Jr., for helpful comments. Amino acid analysis and sequencing was kindly performed by Drs. Graham Hughes and Sérène Frutiger, Department of Medical Biochemistry, Geneva. We also thank Kathy Walker, Sunil Patel, and Dr. Gary McKnit of Zymogenetics, Seattle, WA, for synthesis and mass spectrometry analysis of human C-peptide and human des(27–31)-C-peptide.