Role of the Connecting Peptide in Insulin Biosynthesis*

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In single-chain insulins (SCIs), the C terminus of the insulin B-chain is contiguous with the N terminus of the A-chain, connected by a short bioengineered linker sequence. SCIs have been proposed to offer potential benefit for gene therapy of diabetes (Lee, H. C., Kim, S. J., Kim, K. S., Shin, H. C., and Yoon, J. W. (2000) Nature 408, 483–488) yet relatively little is known about their folding, intracellular transport, or secretion from mammalian cells. Because SCIs can be considered as mutant proinsulin (with selective shortening of the 35-amino acid connecting peptide that normally includes two sets of flanking dibasic residues), they offer insights into understanding the role of the connecting peptide in insulin biosynthesis. Herein we have explored the relationship of the linker sequence to SCI biosynthesis, folding, and intracellular transport in transiently transfected HEK293 or Chinese hamster ovary cells or in stably transfected AtT20 cells. Despite previous reports that direct linkage of B- and A-chains produces a structure isomorphous with authentic two-chain insulin, we find that constructs with short linkers tend to be synthesized at lower levels, with a significant fraction of molecules exhibiting improper disulfide bonding. Nevertheless, disulfide-mispaired isoforms from a number of different SCI constructs are secreted. While this suggests that a novel folded state goes unrecognized by secretory pathway quality control, we find that misfolded SCIs are detected at higher levels in Chinese hamster ovary cells with artificially activated unfolded protein response mediated by inducible overexpression of active ATF-6. Such a maneuver allows analysis of more seriously misfolded mutants with further shortening of the linker sequence or loss (by mutation) of the insulin interchain disulfide bonds.

Insulin, a peptide hormone involved in carbohydrate and lipid metabolism, consists of a 30-residue B-chain and 21-residue A-chain. Although information contained in the sequence of these chains is sufficient for formation of the native molecule (1, 2), insulin is normally synthesized in pancreatic β cells as a longer, single-chain prohormone (proinsulin) in which the B- and A-chains are linked via the “connecting peptide” (C-peptide), a 31-amino acid sequence flanked by dibasic amino acids (3). Thus by definition within proinsulin, the C-peptide brings the B- and A-chains together such that initial folding and disulfide bond formation in the endoplasmic reticulum (ER) can occur intramolecularly (4). However it has been shown that, at least in vitro, simply linking the B- and A-chains together sequentially, without any C-peptide, also will result in a normally folded “miniproinsulin” (5, 6). It can be argued alternatively that the primary importance of the C-peptide spacer sequence is that it provides suitable flexibility for presenting the dibasic cleavage sites (4) for endoproteolysis by prohormone convertases (7). Such cleavage is of enormous biological significance, since an insulin-like molecule in which the N terminus of the A-chain is tethered directly to the C terminus of the B-chain is devoid of activity on insulin receptors (because of inability of the B-chain C terminus to be displaced, thereby disturbing proper contact of other key residues with the receptor). Nevertheless, the fact that such a bioengineered “direct linkage” construct can traverse the secretory pathway of AtT20 cells, become stored in granules, and be released to the medium upon secretagogue stimulation (8), in conjunction with the aforementioned findings (5, 6), would seem to argue that a linker peptide is dispensable at least for achieving a native insulin protein structure.

Single-chain insulins (SCIs) are insulin analogs in which the C-peptide of proinsulin has been replaced by an artificial linker peptide whose length and sequence is controlled by bioengineering. Unlike the direct linkage construct noted above, some SCI constructs clearly have potent biological activity on insulin receptors (9), making these constructs of potential interest for gene therapy of type 1 diabetes where a replacement insulin gene might encode a product that does not require endoproteolytic activation for expression in non-β cells. (Such genetic manipulation seems reasonable given that C-peptide of proinsulin is not particularly well conserved during evolution (10) and is shorter in guinea pig by two residues, in duck by five residues, and in dog by eight residues (11)). However, to our knowledge, there is only very limited information regarding the folding of SCIs in the context of the ER leading to intracellular transport and secretion from mammalian cells.

In the course of recent studies on the role of B-chain point mutations in insulin trafficking in the secretory pathway (12), we were surprised to discover that H10D, a point mutation that is known to improve the thermodynamic stability of insulin, nevertheless causes insulin disulfide bond mispairing in the ER of mammalian (and yeast) cells, underscoring differences in disulfide bond formation in vitro and in vivo (13, 14). In the current study, we have expanded our analysis to re-examine the role of the linker peptide on insulin biosynthesis. The evidence to be presented suggests that, contrary to previous
beliefs, there is a requirement for a linker peptide in order for insulin to form all three native disulfide bonds in mammalian cells in vivo. As the presence of these disulfide bonds is required for full insulin potency (15, 16), such an investigation provides new guidance for the design of SCIs for gene therapy of diabetes and also sheds light on the role of the C-peptide in insulin biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Other Materials—**Polyclonal anti-insulin was made in guinea pigs (Linco Research, St. Charles, MO). Secondary antibodies and peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA); Zysorbin was from Zymed Laboratories Inc., and protein reductant A (also known as Zysorbin) was from Sigma. [75S]methionine and [35S]methionine/cysteine mixture (Expera [59x35]35S) were purchased from New England Nuclear (New Bedford, MA). Methionine/cysteine-deficient mammalian cell culture media, brefeldin A, and stock chemicals were from Sigma.

**Linker Sequence Mutagenesis—**The pRSV-NeoDC plasmid (encoding a preproinsulin mutant in which the B-chain is directly contiguous with the A-chain, lacking any C-peptide) was obtained from Dr. H. P. Moore (University of California, Berkeley, CA), and this was subcloned to pcDNA3 and was used as double-stranded DNA template. All of the artificial linker sequences or other point mutations were prepared by PCR mutagenesis using the four-primer method. In brief, one primer pair encodes the restriction site at one end and the desired mutation sequence at the other end. The second primer pair contains the desired mutation sequence encoded on the reverse orientation primer (21–24-base overlap in sequence with the first pair) and encodes a second restriction site at the opposite end. The PCR products of the first two rounds of PCR were then used as template for a final PCR reaction using the external flanking primers encoding the restriction sites, and this product was subcloned initially into the pGEM T-vector, sequenced for confirmation of each mutation, and finally subcloned into the pcDNA3 expression vector.

**Mammalian Cell Culture and Transfection—**CHO-Lac cells (originally called CHO-3.6 Lac, which stably express the Lac-transactivator under control of the CMV immediate-early promoter) were obtained from Dr. M. G. Roth (University of Texas Southwestern Medical Center, Dallas, TX) (17) and maintained in the same medium plus 200 μg/ml hygromycin. Two plasmids, called pCGLATF6-1790 (terminal HA tag) and pCGNATF6-1790, were obtained from Dr. R. Prywes (Columbia University, New York, NY). The first of these plasmids encodes the peptide MASSYYDVPYDIALGGPSR containing a single HA tag (underlined) immediately upstream of ATF6 residue 1 with a stop codon following residue 373 and a 3′-BamHI site beginning nine nucleotides downstream. However, upon sequencing of this construct we identified a point mutation encoding a M313I substitution. To correct the point mutation, we used pCGNATF6-1790 as template and PCR-amplified a fragment containing an internal KpnI site and a newly engineered BamHI site from amino acid 373 followed by BamHI, and we used this amplified product to replace the KpnI-BamHI fragment of ATF6-373 (terminal HA tag). Another PCR was performed to add 5′-NotI and 3′-XhoI restriction sites flanking the insert. Finally, a NotI-XhoI digest allowed subcloning the corrected ATF6-373 (terminal HA tag) into pCMV3RLac in which the luciferase insert was excised by NotI-XhoI digestion. This plasmid contains 450 bases of repeated Lac repressor binding sites immediately downstream of the CMV promoter and upstream of the inserted cDNA of interest. The final plasmid called pCMV3.R.HA-ATF6 was transfected into CHO-Lac cells, and stable clones were selected with G418 at 800 μg/ml. The clone used in these experiments, CLA14, is characterized primarily on the length of the linker peptide contained within the A-chain (19–21).

**RESULTS**

**Abnormal Nonreducing SDS-PAGE Mobility for Single-chain Insulin Bearing a Short Linker Sequence—**It has been reported that the insulin B-chain guides folding and disulfide bond formation within the A-chain (19–21). Nevertheless, we found surprising the recent result that certain B-chain point mutations, including ones known to provide thermodynamic stability to insulin that has been folded in vitro (such a substitution of insulin HisB10 (Asp), induce insulin disulfide mispairing within the secretory pathway in vivo (12). In this study, we have primarily used analysis by nonreducing SDS-PAGE to assess insulin disulfide maturation, which is generally recognized to occur within the endoplasmic reticulum (22), focusing primarily on the length of the linker peptide contained within SCIs. In an initial series of constructs, we created “artificial C-peptides” from five to nine residues in which the linker sequence begins and ends with a Met residue, flanking a stretch of glycine residues. When these SCI constructs were expressed in transiently transfected 293 cells, they exhibited nearly ideal behavior under nonreducing and reducing conditions, although the migration was slower (higher) under reducing conditions, reflecting a more open rod-like conformation expected in SDS-PAGE upon dissolution of the disulfide bonds (right half of each gel shown in Fig. 1). We prepared additional constructs in which the linker sequence was foreshortened either to four, three, or two Gly residues or in which the B-chain had direct linkage to A-chain with no linker whatsoever. The SCI constructs with short Gly-containing linkers showed diminished incorporation of [35S]Met/Cys label at least in part because of absence of methionines from the linker sequence; however, it was clear that these constructs were also synthesized at low levels, as indicated by the fact that even direct linkage of B- and A-chains with no C-peptide showed a reproducibly stronger signal upon transient expression (left half of each gel shown in Fig. 1). Moreover, while each of the foreshortened linker constructs showed nearly ideal behavior under reducing conditions, they showed a far more complex pattern, with more than one band of anomalous mobility (indicating non-ideal behavior) under nonreducing conditions (Fig. 1). These data are highly reminiscent of disulfide mispairing observed upon introduction of B-chain point mutations in SCIs or proinsulin (12). Although not

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**Metabolic Labeling, Immunoprecipitation, and Tricine-Urea-SDS-PAGE—**Cells were metabolically radiolabeled with [75S]methionine/cysteine mixture in methionine/cysteine-deficient medium and chased in complete medium for the times indicated. Where indicated, brefeldin A (BFA) was included during labeling and chase at a concentration of 10 μg/ml. At the end of the chase, medium was collected and the cells were lysed in 100 mM NaCl, 0.2% Triton X-100, 0.1% SDS, 10 mM EDTA, and 25 mM Tris, pH 7.4. Cell lysates and chase media were treated with a proteinase inhibitor mixture (Roche Applied Science, Indianapolis, IN), precleared with Zysorbin (Zymed Laboratories Inc.) for 30 min and then subjected to immunoprecipitation with guinea pig anti-insulin plus additional Zysorbin. Immunocomplexes were sedimented at 12,000 × g for 1 min, and pellets were washed twice with methanolysis buffer and once with buffer (0.5 mM NaCl, 1% Triton X-100, 10 mM EDTA, and 25 mM Tris, pH 7.4). The samples were then analyzed by Tricine-urea-SDS-PAGE system (18) with previously noted modifications (12); samples were allowed to enter the gel at 50 V for 1 h and then run at 100 V overnight without cooling. (As described previously, the gel solutions were not routinely de-gassed before use (12); however, after the experiments for this paper were completed we discovered that a small but variable degree of thiol reoxidation can occur in vitro for our samples during the running of the gel itself, and this can be effectively decreased by de-gassing the gel solutions.) Insulin gels were fixed initially in 20% trichloroacetic acid without alcohol, then in 12.5% trichloroacetic acid plus 50% methanol, then incubated briefly with water, and finally either phosphorimaged or incubated with 1M sodium salicylate for 20 min and exposed to XAR film at ~70°C.
Each well was pulse-labeled with 35S-labeled amino acids and chased. Connecting peptide links B- and A-chains is termed encoding SCIs with the linker sequences shown. An SCI in which no pcDNA3 containing either no cDNA insert (Empty Vector) or cDNAs encoding SCIs with the linker sequences shown. An SCI in which no connecting peptide links B- and A-chains is termed Direct Linkage. Each well was pulse-labeled with 35S-labeled amino acids and chased for 1 h. The cells were lysed, and both lysates and chase media were immunoprecipitated with anti-insulin. Each immunoprecipitate was divided in two, and each half was analyzed by SDS-PAGE either under nonreducing conditions (left gel) or after reduction with 20 mM dithiothreitol (right gel) followed by fluorography. In this figure, only immunoprecipitates from the chase media are shown; however, the pattern of bands was identical from the cell lysates. For comparison, a nonreduced radioiodinated insulin standard is shown in the first lane.

Exhaustively explored in this study, when the linker was short, small sequence changes did affect the nonreducing SDS-PAGE mobility of the predominant isoform of insulin. As shown in Fig. 2, an MGGM tetrapeptide linker resulted in most SCI molecules migrating with a mobility that was only slightly slower (higher) than authentic insulin (either biosynthetically labeled from βT3-3 cells or iodinated standard), whereas an SCI with the GGGG tetrapeptide linker (in addition to being synthesized weakly) was higher still, despite a virtually identical molecular mass.

On the one hand, some of the slower nonreducing SDS-PAGE mobility of SCIs could be directly attributed to the presence of the linker peptide, as demonstrated by an experiment in which an immunoprecipitated SCI bearing a nine-residue linker sequence was either partially digested or not digested with CNBr (Fig. 3). Without CNBr digestion, the reduced SCI shifts up (to a slower mobility), precisely as in Fig. 1. After partial CNBr digestion, two bands are recovered upon nonreducing SDS-PAGE: the upper band precisely co-migrates with the undigested species, while the lower band precisely co-migrates with the two-chain insulin standard (lane marked I). Upon reduction with DTT, the upper band co-migrates with undigested SCI shifts up whereas the lower band co-migrates with two-chain insulin shifts down (in Fig. 3, the identical gel exposure is shown twice; on the left are the original data, while the right shows superimposed arrows clarifying this interpretation). The upward shift of the upper band is consistent with disulfide reduction allowing the uncleaved SCI protein to assume a rod-like open conformation, whereas the downward shift of the lower band is consistent with dissociation of the two chains from the cleaved SCI (as is also shown in Fig. 3 for authentic insulin standard). Thus, for SCIs shown with linkers longer than four or five residues (such as in Fig. 3), the difference in mobility on nonreducing SDS-PAGE from that of authentic two-chain insulin can be attributed entirely to the presence of the linker itself, without invoking any insulin structural perturbation. On the other hand, for direct linkage of the B- and A-chains (Fig. 2), it appeared that the slow (high) mobility observed for the major species could only be explained by disulfide malformation.

Single Chain Insulins Missing Individual Disulfide Pairs—We considered the possibility that upon shortening the C-peptide, one or more insulin disulfide bonds might fail to form (resulting in free thiols) versus actual disulfide mispairing. Previously we found that introduction of selected B-chain point mutations caused similar abnormal upward mobility shifting upon nonreducing SDS-PAGE but without reactivity to alkylating agents that modify free thiols (12). In this report, using an SCI with a seven-residue linker as template, we elected to individually mutate each of the three conserved cysteine pairs in the hopes of identifying their relative contributions to protein biosynthesis, secretion, and especially to nonreducing SDS-PAGE mobility. Loss of the insulin disulfide bond between the sixth and seventh positions of the A-chain by

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**Fig. 1.** Anomalous nonreducing SDS-PAGE mobility of SCI constructs bearing a highly foreshortened connecting peptide. Identical wells of HEK293 cells were transiently transfected with 2 µg pcDNA3 containing either no cDNA insert (Empty Vector) or cDNAs encoding SCIs with the linker sequences shown. An SCI in which no connecting peptide links B- and A-chains is termed Direct Linkage. Each well was pulse-labeled with 35S-labeled amino acids and chased for 1 h. The cells were lysed, and both lysates and chase media were immunoprecipitated with anti-insulin. Each immunoprecipitate was divided in two, and each half was analyzed by SDS-PAGE either under nonreducing conditions (left gel) or after reduction with 20 mM dithiothreitol (right gel) followed by fluorography. In this figure, only immunoprecipitates from the chase media are shown; however, the pattern of bands was identical from the cell lysates. For comparison, a nonreduced radioiodinated insulin standard is shown in the first lane.

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**Fig. 2.** Distinct anomalous nonreducing SDS-PAGE band mobilities for two constructs bearing a linker sequence of identical (four-residue) length but differing sequence. HEK293 cells were transfected with cDNAs encoding SCIs bearing the linker sequences shown or with the empty expression vector. An SCI in which no connecting peptide links B- and A-chains is termed Direct Linkage. The transfected cells were then analyzed exactly as in Fig. 1. Only nonreducing SDS-PAGE (followed by fluorography) is shown. A radioiodinated insulin standard and an anti-insulin immunoprecipitate from 35S-labeled amino acid labeled βTC-3 cells are shown at left to identify the SDS-PAGE mobility of proinsulin and insulin.

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**Fig. 3.** Changes in mobility of uncleaved and cyanogen bromide-cleaved SCI as a consequence of disulfide bond reduction. A seven-lane gel bearing six samples (and one blank lane) is shown twice in exact repeat. The lanes marked I contain radioiodinated insulin standard. An immunoprecipitated, metabolically labeled SCI (secreted from transfected HEK293 cells; linker sequence shown at bottom) is analyzed by SDS-PAGE under nonreducing conditions or reduced with 20 mM DTT in the third and sixth lanes, respectively. For CNBr-mediated cleavage of approximately half of the molecules, the immunoprecipitate was treated with 50 mg/ml CNBr in 6 M guanidine plus 20 mM HCl at room temperature overnight, and this was then was spun through a Microcon YM-3 ultrafilter (Millipore, New Bedford, MA) at 10,000 x g for 50 min to de-salt the sample before mixing with SDS-gel sample buffer. After CNBr treatment, the SCI band can be seen to be split in two under nonreducing conditions: half co-migrating with the original uncleaved SCI and the other half co-migrating with the insulin standard. Upon reduction of the CNBr-digested sample, approximately half the molecules shift up in the gel, co-migrating with the reduced SCI exhibiting diminished SDS-PAGE mobility that indicates lack of peptide chain cleavage, while the other half shifts down in the gel, co-migrating with the insulin standard in which the interchain disulfide bonds have been reduced to release two chains, indicating that this faster-migrating SCI band has been proteolytically cleaved by CNBr.
and 2). In pulse-chase experiments with 35S-labeled amino acids for 30 min and chased for 1.5 h, the cells were lysed, and both lysates and chase media were immunoprecipitated with anti-insulin. The numbering of residues in the construct begins with residue 1 of the B-chain; the mutant C43S/C48S indicates loss of the A6-A11 disulfide. B, CLA14 cells (see "Experimental Procedures") were pulse-labeled with 35S-labeled amino acids for 30 min and chased for 40 min. The cells were lysed, and both lysates (C) and chase media (M) were immunoprecipitated with anti-insulin (note that the order in which the cell and media lanes were loaded on the gel differ from that shown in panel A). An arrowhead at right marks the position of the anomalously migrating SCI consequent to loss of the B19-A20 bond (C19S, C56S). At later chase times also (not shown), this construct remains defective for secretion to the medium.

FIG. 4. Nonreducing SDS-PAGE mobility of SCIs bearing a seven-residue linker either lacking the A6-A11 intrachain- or B19-A20 interchain-disulfide bond. A, CHO cells were pulse-labeled for 1 h with 35S-labeled amino acids and chased for 1.5 h; the cells were lysed, and both lysates and chase media were immunoprecipitated with anti-insulin. The numbering of residues in the construct begins with residue 1 of the B-chain; the mutant C43S/C48S indicates loss of the A6-A11 disulfide. B, CLA14 cells (see "Experimental Procedures") were pulse-labeled with 35S-labeled amino acids for 30 min and chased for 40 min. The cells were lysed, and both lysates (C) and chase media (M) were immunoprecipitated with anti-insulin (note that the order in which the cell and media lanes were loaded on the gel differ from that shown in panel A). An arrowhead at right marks the position of the anomalously migrating SCI consequent to loss of the B19-A20 bond (C19S, C56S). At later chase times also (not shown), this construct remains defective for secretion to the medium.

mutation of these Cys residues to Ser (C43S, C48S) caused no loss of recombinant protein production, no loss of recombinant protein secretion and no detectable mobility shift of the predominant insulin species identified by nonreducing SDS-PAGE (Fig. 4A). By contrast, deletion of the disulfide normally existing between B-chain residue 19 and A-chain residue 20 (Fig. 4B, C19S, C56S) caused decreased production of the recombinant protein, being retained intracellularly rather than being secreted, as well as a slower (higher) SCI band mobility by nonreducing SDS-PAGE. The foregoing data demonstrate that presence of the intra-A-chain disulfide bond is not required for formation of the fast-migrating compact form detected electrophoretically nor in secretion of the SCI; however, it must be emphasized that this does not exclude that A6 or A11 might be involved in disulfide mispairing for SCIs bearing a foreshortened C-peptide (23). By contrast, the B19-A20 disulfide bond is important for protein biosynthesis, formation of the compact form detected electrophoretically, and secretion. (As described further below), the B7-A7 disulfide bond is also important for protein biosynthesis and secretion.)

Misfolded SCIs Traverse the Secretory Pathway of Mammalian Cells That Have No Known Defects in Secretory Pathway Quality Control—We examined secretory kinetics both for an SCI bearing a seven-residue linker and apparently normal disulfide bonding and for an SCI bearing direct linkage of B- and A-chains with apparent disulfide mispairing (see Figs. 1 and 2). In pulse-chase experiments with 35S-labeled amino acids, both newly synthesized constructs showed an apparent secretory half-time of 30 min (Fig. 5A). At 3 h of chase, ~90% of both constructs had been secreted, whereas no secretion of either construct was detected at this time in cells treated with brefeldin A (Fig. 5B). The data indicate that both the properly and improperly disulfide bonded forms of SCI traverse the secretory pathway of 293 cells. Identical results were also obtained in CHO cells (and AtT20 cells, see below).

Proof That an Insufficient Linker between B- and A-chains Results in a Predominant Species with Mispaired Disulfide Bonds, Corrected by Re-folding in Vitro—Several SCI constructs bearing different linkers were exposed to in vitro disulfide reshuffling conditions using a mixture of reduced and oxidized glutathione at a 10:1 ratio. In Fig. 6A, an SCI with a nine-residue linker (that is potently bioactive on insulin receptors)3 was compared with direct linkage of B- and A-chains. After exposure to the glutathione mixture, the SCI with a nine-residue linker showed relatively modest changes in mobility upon nonreducing SDS-PAGE, whereas for the direct linkage, the major species refolded to a form with a substantially faster gel mobility. This downward mobility shift of the directly linked chains does not reflect a proteolytic cleavage between B- and A-chains because upon re-reduction of the product with 100 mM DTT the predominant species once again shifted back to a slower migrating form, similar to the behavior of the control SCI with a nine-residue linker (Fig. 6A, last two lanes). A modification of the direct linkage construct was also tested in which Thr-30 of the B-chain was replaced with methionine. (Cleavage with C8NBr to yield a two-chain form of the B30Met direct linkage construct (not shown) yields a nonreduced band with persistently slower mobility from that of authentic two-chain insulin despite virtual amino acid identity providing further confirmation that the anomalous gel mobility stems from disulfide mispairing.) The T30M mutation caused essentially no change in the dramatic ability of the direct linkage construct to refold its disulfide bonds to the more compact form or to reduce them subsequently with DTT (Fig. 6B). In addition, as shown in Fig. 6B, disulfide reshuffling conditions did not show such dramatic mobility changes for the SCI bearing a seven-residue linker with deletion of the A6-A11 disulfide bond (a construct first introduced in Fig. 4A), further supporting the idea that the two interchain disulfide bonds formed properly despite absence of the intra-A-chain disulfide.

One might expect that unlike the disulfide reshuffling conditions provided by a mixture of reduced and oxidized glutathione, creation of free sulfhydryl groups in SCIs by reduction with DTT, followed by addition of iodoacetamide (IAA) to irreversibly modify those sulfhydryls, would result exclusively in reduced and carboxymethylated SCIs with no possibility of disulfide re-formation. However, for some cysteine-containing polypeptides it has been shown that upon addition of alkylating agent, thiol-disulfide rearrangement occurs on the same time scale as alkylation; thus, a kinetic competition exists between the two reactions (24, 25). Indeed, we recently reported that in vitro reshuffling of insulin disulfide bonds in some SCIs occurs for a fraction of the molecules upon adding IAA immediately after DTT treatment (12). In the main gel of Fig. 7, cells bearing either the direct linkage construct or the presence of four- or seven-residue linkers underwent pulse-chase (1 h label, 1 h chase) with both cell lysate and chase media collected.

3 M. Liu and P. Arvan, manuscript in preparation.
reduced (3 mM IAA for 30 min at 37 °C) HEK293 cells were transfected with cDNAs encoding SCI disulfide interchange to a state of improved disulfide pairing from that observed in vivo as measured by nonreducing SDS-PAGE mobility. 35S-labeled SCIs bearing the linker sequences shown were secreted and immunoprecipitated with anti-insulin (an aliquot taken, first two lanes) and were then exposed to a reduced/oxidized glutathione mixture in a 10:1 ratio (an aliquot taken, middle two lanes) and finally reduced with 100 mM DTT (last two lanes). A, upon direct linkage of B- and A-chains, despite a markedly slower initial SDS-PAGE mobility of the major isoform, this construct actually exhibits a faster gel mobility than the control (nine-residue linker) construct after exposure to in vitro disulfide reshuffling conditions. B, a similarly anomalous band mobility and dramatic disulfide isomerization is observed when B30Thr is replaced by methionine in the direct linkage construct. A second construct contains the seven-residue linker sequence shown plus absence of the A6-A11 intrachain disulfide bond (C43S, C48S); such a construct shows more modest band mobility shift upon glutathione-mediated disulfide reshuffling. While the production of the SCI bearing a four-residue (-GGGG-) linker was poor as before, the protein encoded by a construct after exposure to 50 mM IAA either without prior reduction or after initial reduction by boiling in SDS-gel sample buffer containing 20 mM DTT, and this was followed by incubation with 50 mM IAA either without prior reduction or after initial reduction by boiling in SDS-gel sample buffer containing 20 mM DTT. In a separate experiment shown in lanes 15 and 16, an SCI bearing no connecting peptide (Direct Link) that had undergone the reduction/alkylation protocol (DI, identical to lane 4) was then further reduced (→ D) with 100 mM DTT prior to SDS-PAGE and fluorography.

Fig. 7. SCI disulfide reshuffling during a standard reduction-alkylation protocol. HEK293 cells were transfected with cDNAs encoding SCIs bearing the linker sequences shown and were then pulse-labeled with 35S-labeled amino acids and chased as described in Fig. 1. Immunoprecipitates with anti-insulin from cells (C) and media (M) were then exposed to 50 mM IAA either without prior reduction or after initial reduction by boiling in SDS-gel sample buffer containing 20 mM DTT. In a separate experiment shown in lanes 15 and 16, an SCI bearing no connecting peptide (Direct Link) that had undergone the reduction/alkylation protocol (DI, identical to lane 4) was then further reduced (→ D) with 100 mM DTT prior to SDS-PAGE and fluorography.

Aspects of the ER Environment in the Biosynthesis/Secretion of SCIs—Recently we showed that there is a poor correlation between thermodynamic stability of the mature SCI protein and the formation of mispaired disulfide isomers during SCI folding in the ER. There are many possible explanations for this, including the incomplete availability of the entire polypeptide for the folding that occurs co-translationally (26), the unknown kinetic relationship(s) between initial disulfide bond formation and insulin signal peptide cleavage (which can affect subsequent folding of mature polypeptides (27)), and the possible role of ER-specific thiol-interactor proteins that can influence disulfide bond formation (28). In addition one might postulate that because mispaired disulfide isomers are relatively rapidly secreted (Fig. 7), there might be insufficient time in the ER for proper disulfide isomerization of SCIs. We considered this unlikely because constructs with a suitable linker length show proper SCI disulfide isomerization despite nearly identical secretory kinetics (Fig. 5A); nevertheless, we chose to examine the effect of extending the residence of SCIs in the ER with BFA. For both a seven-residue linker bearing a substitution of serine B9 with Asp (12), as well as for direct linkage of B- and A-chains, retention in the ER for up to 6 h of chase did not detectably convert the major disulfide-mispaired isoform into a more compact disulfide-bonded isoform (Fig. 8). Thus the fact that such isoforms are misfolded as judged by disulfide mispairing does not preclude their stability (21), which is consistent with their lack of detection by ER retention machinery or other secretory pathway quality control (Figs. 5 and 7). Nevertheless, it seemed of interest to know if manipulation of the unfolded protein response pathway might influence the biosynthesis or secretion of SCIs with (or without) disulfide mispairing.

To begin to explore this question, we created a stable CHO cell line in which to induce expression of the active ATF6 transcription factor that drives ER chaperone expression in the absence of any ER stress. Normally during ER stress, the ~90-kDa integral membrane form of ATF6 migrates to the Golgi complex (29, 30) and is proteolysed (31) to release an ~50-kDa cytosolic domain containing the leucine zipper motif,
which translocates to the nucleus and activates transcription of ER chaperones and folding enzymes directly (32, 33) as well as indirectly via XBP-1 (34, 35). However, cDNA-mediated expression of recombinant cytosolic ATF6 domain constitutively activates transcription of these (and other) genes without ER stress (36). We therefore engineered an HA-tagged ATF6cyt into the pCMV3R plasmid for IPTG-inducible expression in CLA14 cells (see “Experimental Procedures”). After a 2-day exposure with 15 mM IPTG, the cells express a specific Western-blotting ~50-kDa HA-ATF6cyt band, and without any added ER stress, this leads to expression of a representative ER molecule chaperone (GRP94) at a level exceeding that obtained from cells treated overnight with 15 μg/ml tunicamycin (Fig. 9A).

Cells exposed to inducer for 1 d were then transfected with an expression plasmid encoding either SCI bearing direct linkage of B- and A-chains or containing the same seven-residue linker plus an S9D point mutation shown in Fig. 8. Although these mutant constructs are ordinarily well expressed (Fig. 8), 1 d after transfection (and 2 d after IPTG treatment) there was an obvious further enhancement in the biosynthesis of both constructs (Fig. 9B). The CLA14 cells will be more extensively characterized elsewhere, but as SCI production is not affected in other cell lines treated with IPTG (not shown), the foregoing results suggest that induced expression of active ATF6cyt either increases the efficiency of expression after stress associated with transfection (in effect, increasing transfection efficiency) or does not change transfection efficiency but increases recombinant SCI protein biosynthesis in transfected cells. Regardless of the mechanism (which is still under investigation), this tool allowed us not only to increase the levels of constructs that are already well expressed under ordinary conditions but also permitted detection of SCI constructs that ordinarily were undetectable. Not surprisingly in Fig. 10, an SCI bearing wild-type insulin chains and a seven-residue linker is well synthesized/secreted, and this is increased further in CLA14 cells after induced expression of active ATF6cyt. More remarkably, an SCI with the same linker but in which the B7-A7 interchain disulfide bond has been deleted (by double point mutation of these Cys residues to serine) resulted in a protein produced at extremely low levels and not detectably secreted; while induced expression of active ATF6cyt did not rescue secretion of the construct it nevertheless dramatically increased protein production from transient transfection (Fig. 10, last lane). The low biosynthetic level for this construct was reminiscent of that seen upon deletion of the B19-A20 interchain disulfide bond (indeed, induced expression of active ATF6cyt was employed for experiments with this mutant shown in Fig. 4B); thus it is apparent that the presence of both interchain disulfide bonds are essential to escape secretory pathway quality control. However, only loss of the B19-A20 (and not the B7-A7) disulfide caused significantly slower mobility of the SCI band by nonreducing SDS-PAGE.

Further Foreshortening of the SCI Linker—The foregoing results challenge the assumption that no linker peptide is required for proper folding of insulin in vivo, thereby raising questions about the in vivo validity of in vitro observations suggesting that further foreshortening of the linkage between B- and A-chains, such as deletion of B30Thr or deletion of this residue in addition to B29Lys, would nevertheless permit insulin to be synthesized into a structure essentially isomorphous with authentic two-chain insulin (e.g. Ref. 5). We decided to test this question in the context of SCI folding in the mammalian ER. As these constructs were synthesized at low levels under ordinary conditions, we elected to use CLA14 cells in which ATF6 was induced with IPTG. As shown in Fig. 11, both a B29-A1 direct linkage and a B28-A1 direct linkage construct were produced and secreted from these cells. However, the B29-A1 construct, like the B30-A1 direct linkage construct, showed only a minor fraction of molecules with normal or near-normal mobility by nonreducing SDS-PAGE; instead, the predominant form migrated with abnormally slow gel mobility indicative of a mispaired disulfide-bonded monomeric isomer (which nevertheless was secreted). Even more remarkable was the B28-A1 direct linkage, in which virtually no monomeric isoform could be detected. Instead, almost all of the SCI protein was recovered as a disulfide-linked homodimer (Fig. 11, nonreduced gel), which ran ideally as a monomer upon SDS-PAGE.
production and shows worsening of disulfide bond pairing in vivo. CLA14 cells induced to express ATF6 by with IPTG were transfected with cDNAs encoding the five distinct SCI constructs shown and the next day were pulse-labeled with 35S-labeled amino acids for 1 h and chased for 1 h. SCIs immunoprecipitated from both cell lysates (C) and chase media (M) with anti-insulin were analyzed by SDS-PAGE under nonreducing conditions or after reduction with 100 mM DTT followed by fluorography. Note that while all the constructs exhibit ideal behavior under reducing conditions, both constructs linking B30-A1 showed similarly anomalous nonreducing SDS-PAGE mobility to that of B29 linked to A1, while the B28-A1 linkage results in near-exclusive formation of disulfide-linked SCI dimers.

under conditions reduced with 100 mM DTT (Fig. 11, reduced gel). These data strongly indicate that direct linkage of the B- and A-chains synthesized in vivo tends to result in structures that are not isomorphous with two-chain insulin (due to disulfide mispairing), and further shortening of the linkage between the chains makes matters worse, decreasing protein biosynthesis and more seriously impairing the nascent monomer folding pathway.

SCI Folding in AtT20 Cells—Because direct B- and A-chain linkage does not preclude normal disulfide bond formation in vivo but does impair this process within the ER of HEK293 cells and CHO cells, we did consider the possibility that the profile of ER luminal chaperone activities (37) in cells with a classical regulated secretory pathway might be different, and this might alter the folding outcome. Indeed it has been shown that upon expression in AtT20 cells, an SCI with direct linkage of B- and A-chains is transported efficiently through the secretory pathway and also is stored in secretory granules with an efficiency that is as high or higher than that of authentic insulin, raising the question of whether the construct does or does not fold properly in such cells (8). We therefore introduced into AtT20 cells by transient transfection SCI constructs bearing a seven-residue linker that has normal nonreducing SDS-PAGE mobility (Fig. 1), the complete C-peptide and cleavage sites (i.e. proinsulin), or direct linkage of the chains. With a 60-min pulse and no chase (Fig. 12), each of the constructs was synthesized, and little had yet been secreted to the medium (although all constructs were eventually secreted, not shown).

Importantly, absence of a C-peptide caused the same formation of mispaired disulfide isomers in these cells, with a mobility upon nonreducing SDS-PAGE mid-way between that of the seven-residue linker and authentic proinsulin (Fig. 12). These data support the previous presumption that direct linkage of B- and A-chains are misfolded in vivo, but despite this, the construct forms a sufficiently intact three-dimensional structure to escape secretory pathway quality control (8).

**DISCUSSION**

Although information contained solely within the B- and A-chains of insulin is sufficient for insulin protein structure (1, 2), a previous report examining the apparently normal intracellular trafficking of a construct called InsDC (i.e. B- and A-chains directly linked with no C-peptide) was not clear about whether the insulin moiety is properly or improperly folded within the secretory pathway of mammalian cells (8). This matter was seemingly irrelevant to insulin action because it has long been clear that such a construct, even if the disulfide bonds are correctly assembled, is almost completely unable to bind insulin receptors (5, 6, 38). However, in light of recent studies indicating that SCI constructs may have utility in gene therapy of type 1 diabetes (9), it now seems surprising that there is a paucity of information about issues relating to biosynthesis, folding, and secretion of SCIs from mammalian cells. In the present study, we have endeavored to determine to explore just one aspect of this question, namely, the role of a connecting peptide in insulin folding and intracellular transport in the secretory pathway.

Our data lead to the conclusion that a C-peptide is required for proper insulin folding in the ER. Specifically, although the possible number and combination of bioengineered amino acids comprising such a linker is endless and conclusions cannot be drawn about sequences that have never been tested, from our previous and current studies using a simple set of constructs we suggest that insulin disulfide bonds form early during protein folding in the ER (22) with subpopulations of disulfide isomers that are not readily interconverted thereafter (Fig. 8 and Ref. 12). A minimum linker length of five residues is needed to facilitate formation of the proper disulfide isomer (Figs. 1 and 2). While only a small slowing of electrophoretic mobility can be directly attributed to the presence of the linker itself (Fig. 3), loss of the proper disulfide pairings, and this is proved for the B19-A20 pairing (Fig. 4B), causes loss of compactness as measured by additional slowing of mobility upon SDS-PAGE selectively under nonreducing conditions. The fact that loss of the A6-A11 or B7-A7 disulfide pair causes no discernable slowing of nonreducing gel mobility (Figs. 4 and 10), whereas direct linkage of B- and A-chains clearly does (Figs. 1 and 2), provides strong circumstantial evidence that the gel mobility assay reflects more about the gain of improper insulin disulfide bonds than the loss of normal insulin disulfide bonds. Nevertheless, consistent with previous reports (16), it is likely that the severity of misfolding cannot be correlated solely with the nonreducing SDS-PAGE mobility because direct linkage of B- and A-chains is secreted from cells with near-normal kinetics and efficiency (Fig. 5), whereas loss of either of the disulfide linkages between B- and A-chains causes a profound inhibition of insulin secretion (Figs. 4 and 10). (However, loss of the intra-A-chain disulfide bond is compatible with good protein biosynthesis and secretion, suggesting the least severe folding defect (Fig. 4.) Thus, insulin with mispaired disulfide bonds forms a novel structure or structures capable of escaping secretory pathway quality control, whereas insulin lacking in-
terchain disulfide bonds apparently exposes structural information to the secretory pathway quality control machinery (most likely, ER chaperones (39)) that is recognized by the cell as misfolded. Similar conclusions have been reached about the importance of the interchain disulfide bonds for insulin expressed in the secretory pathway of the yeast, Saccharomyces cerevisiae (40).

Our strongest evidence that the disulfide isomerization occurs within the secretory pathway and does not reflect some artifact of our method of sample analysis comes from the fact that identical sample analysis after exposure of misfolded constructs to in vitro disulfide reshuffling conditions allows for recovery of a faster migrating species upon nonreducing SDS-PAGE, and this faster migrating species can once again be converted to a slower migrating species upon reduction of disulfide bonds (Figs. 6 and 7). That fact that an SCI with a -GGGG- linker migrates more slowly upon full reduction than for an SCI with direct linkage of B- and A-chains and yet both constructs migrate anomalously upon nonreducing SDS-PAGE with the direct linker construct exhibiting an even slower gel mobility strongly implies that more than one possible disulfide mispairing is achieved upon foreshortening of the C-peptide, with different isomers preferentially enriched depending upon the precise length and sequence of the linker (Figs. 1, 2, and 7) as a consequence of structural stress imposed by a tight turn between B- and A-chains (41).

In our pursuit of the question of whether disulfide-mispaired SCI constructs are recognized by mammalian cells as misfolded, we have begun analysis of cells in which the unfolded protein response is artificially activated upon IPTG-inducible expression of active ATF6<sub>cyt</sub> (Fig. 9A). Indeed, such a maneuver increases biosynthesis of most of the constructs studied in this report. Because of the protocol we used, we do not presently know whether this increase in expression reflects primarily improved transfection efficiency of CLA14 cells, improved translation of SCI proteins, or decreased ER-associated degradation. Thus at present, the primary utility of the CLA14 cells is the detection or in the fraction of SCI protein that gets secreted. Thus at present, the primary utility of the CLA14 cells is the detection of secretory proteins that are otherwise difficult to produce.

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