High Affinity Dimerization by Ski Involves Parallel Pairing of a Novel Bipartite α-Helical Domain

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c-Ski protein possesses a C-terminal dimerization domain that was deleted during the generation of v-ski, and has been implicated in the increased potency of c-ski in cellular transformation compared with the viral gene. The domain is predicted to consist of an extended α-helical segment made up of two motifs: a tandem repeat (TR) consisting of five imperfect repeats of 25 residues each and a leucine zipper (LZ) consisting of six heptad repeats. We have examined the structure and dimerization of TR or LZ individually or the entire TR-LZ domain. Using a quenched chemical cross-linking method, we show that the TR dimerizes with moderate efficiency ($K_d = 4 \times 10^{-8} \mathrm{M}$), whereas LZ dimerizes poorly ($K_d > 2 \times 10^{-5} \mathrm{M}$). However, the entire TR-LZ domain dimerizes efficiently ($K_d = 2 \times 10^{-8} \mathrm{M}$), showing a cooperative effect of the two motifs. CD analyses indicate that all three proteins contain predominantly α-helices. Limited proteolysis of the TR-LZ dimer indicates that the two helical motifs are linked by a small loop. Interchain disulfide bond formation indicates that both the LZ and TR helices are oriented in parallel. We propose a model for the dimer interface in the TR region consisting of discontinuous clusters of hydrophobic residues forming “leucine buttons.”

When overexpressed from a retroviral vector, the c-ski proto-oncogene, like its viral homolog v-ski, induces transformation and promotes muscle differentiation in cultured avian fibroblasts (1–3). The c-ski gene has a total of eight coding exons and is alternatively spliced (4, 5). The largest transcript contains all eight exons and encodes an 84-kDa protein (6). The v-ski oncogene is derived from the first five coding exons of c-ski and encodes a 49-kDa protein (7). Surprisingly, the c-Ski protein is a more potent transforming agent than v-Ski as determined by morphological transformation and focus formation assays (1). The stronger biological activity of c-Ski has been attributed to its C-terminal dimerization domain (8), which is missing in v-Ski (5, 7). This domain is predicted to be α-helical and contains two amino acid sequence motifs: an N-terminal motif formed of five tandem repeats of 25 amino acid residues each (9, 10), followed by a C-terminal leucine zipper (LZ) motif consisting of six heptad repeats (10). It has been shown that both motifs are necessary for high affinity homodimerization of Ski and heterodimerization between Ski and the product of a ski-related gene, sno (8).

The tandem repeat motif (TR) has limited sequence relatedness with segments in myosin, intermediate filaments, laminins, and other fibrous proteins that form α-helical coiled coils (9). Amino acid composition analysis shows that alanine (11%), leucine (14%), arginine (12%), lysine (14%), and glutamate (17%) constitute two thirds of the residues in the TR. This composition profile is consistent with that of a leucine zipper coiled coil, which is common among several transcription factors (11, 12). The leucine zipper motif consists of heptad repeats with leucine or other hydrophobic residues at two neighboring positions on the helix of each monomer. These residues form a hydrophobic interface that extends along the long axis of the double helix. However, the predicted TR α-helix of Ski does not have a continuous hydrophobic face. Instead, there is a strong pattern of alternating leucines and charged residues at the heptad positions normally occupied exclusively by hydrophobic residues (9). If the tandem repeat motif does form a coiled coil, it must rely on an as yet uncharacterized interhelical interaction. Moreover, the proposed TR helix involves 125 amino acid residues, stretching over 180 Å in length. Helices of such length are not uncommon among fibrous and muscle proteins but are rare among transcription factors such as Ski (13), having been found in only a single bacterial factor (14). The leucine zipper helices of all other transcription factors consist mostly of five or six heptad repeats (12), such as the leucine zipper immediately downstream of the TR domain in Ski.

We now report the findings of a structure-function study of the Ski dimerization domain. Our aim is to define the roles of the two identified structural elements in dimerization, and particularly to determine whether the tandem repeats form α-helices. For this purpose, polypeptides consisting of the tandem repeat motif, the leucine zipper motif, or both are analyzed by hydrodynamic measurements, chemical cross-linking, circular dichroism spectropolarimetry, and partial proteolysis. In addition, we describe a newly devised quenched cross-linking method to quantify dimerization affinity. Our results provide strong evidence that the tandem repeats and the leucine zipper motifs cooperate in forming a parallel helical dimer. Because the tandem repeat helices do not contain the amphipathic structure of classical coiled coils, we propose a new discontin-
uous dimer interface involving the unique array of residues that constitute the core consensus of the repeat (LXXELEXLIR).

MATERIALS AND METHODS

Constructs and Protein Production—An MslI-XhoI fragment of the chicken c-ski cDNA sequence FB29 (6), which encodes the entire C-terminal dimerization domain of c-Ski from Ser558 to Asn750, was used to generate the coding sequences for the proteins studied. To facilitate cloning and expression, the sequence CCATGGGGGGATC was added to the 5’ (MluI) end of the fragment to produce construct c-ski, coding for the protein called TR-LZ. To generate the construct that encodes only the tandem repeat motif, c-ski was 3’-truncated at a ScaI site. The resultant construct, c-skims, encodes the TR protein, which contains the c-Ski sequence Ser558–Lys844. To generate the construct that encodes only the leucine zipper motif, the MslI-XhoI fragment was 5’-truncated at the same ScaI site. A short sequence CCATGGGGGGATC was attached to the ScaI end to produce construct c-skins, coding for the LZ protein, which contains the c-Ski sequence Ser606–Arg700.

For in vitro translation, c-skim and c-skims were cloned as an NcoI-XhoI fragments at the cognate sites in plasmid 5 EETM1 (a gift from Dennis Tememton). c-skim was cloned as an NcoI-ScaI fragment at NcoI and SacI sites in the same plasmid. 35S-Labeled TR-LZ, TR, and LZ proteins were produced using the reticulocyte lysate TNT-coupled transcription-translation kit of Promega.

For bacterial expression, c-skim and c-skims were cloned into plasmid pET28 and expressed in strain BL21 (DE3) (Invitrogen). c-skim was cloned as an NcoI (filled)-XhoI fragment inserted at NcoI and XhoI sites. It was expressed as a fusion protein containing an N-terminal histidine tag. c-skims was cloned as an NcoI-ScaI fragment inserted at NcoI and XhoI (filled) sites, and the resultant fusion protein contains a C-terminal histidine tag. For bacterial expression of LZ, a polymerase chain reaction fragment was generated using a primer pair CATGCGATGGCCAGAAGTCTCTTGGAGCCAGAT-CAGGACCACAC (pmllzr) and CTGTCGATGGCCGAGCCAGCCAGCGTTTCCT (pmlzn2) and c-skim as template. The fragment was cleaved by NcoI and ScaI and cloned into pET28 at NcoI and XhoI sites. The LZ protein was expressed from this construct contains a C-terminal histidine tag and is flanked by a lysine residue at both N and C termini to facilitate chemical cross-linking and by a cysteine residue at the N terminus for determination of helix orientation. Bacterial cell culture, protein induction, and histidine tag-based protein purification were performed according to the Invitrogen manual. For further purification, the proteins were placed in buffer containing 50 mM NaH2PO4 (pH 8.0), 0.5 M NaCl, 1.4 M (NH4)2SO4, and 5 mM DTT and loaded onto a phenyl-Sepharose column. The column was developed with a linear gradient of 0.5 M NaCl, 1.85 S. Fractions collected from the column or the gradient were analyzed by SDS-PAGE and autoradiography to detect the 35S-labeled sample proteins. For gel filtration, the protein was performed similarly, except that the bacterially produced protein was cleaved by factor Xa.

Sedimentation—Sedimentation of the proteins was determined by the method of Porath (17). The sample was analyzed by SDS-PAGE and autoradiography to detect the 35S-labeled sample proteins. For gel filtration, the protein was performed similarly, except that the bacterially produced protein was cleaved by factor Xa.

Chemical Cross-linking and Cysteine Oxidation—Chemical cross-linking using bis(sulfosuccinimidyl) suberate (BS3) was performed as described (8) with the following modifications. For analysis of the dimerization of in vitro translated TR, BS3 cross-linking was performed at room temperature in buffer containing 25 mM HEPES (pH 7.3), 200 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.01% Nonidet P-40, and 20% glycerol. The reactions were terminated by addition of 100 mM glycine. For analysis of the effect of salt concentration on TR dimerization, in vitro translated TR was dialyzed to about 2 mM and reacted with BS3 in buffer containing 10 mM HEPES (pH 7.3), 1 mM EDTA, 1 mM DTT, and varying amounts of NaCl. Oxidation of in vitro translated TR was performed in 25 mM HEPES (pH 7.3), 200 mM NaCl, 0.5 mM EDTA, 0.01% Nonidet P-40, and 20% glycerol. The protein solution (100 μl) was placed in a 1.7-ml microcentrifuge tube and incubated at 4 °C with the lid open. Samples (10 μl) were taken at intervals of 1, 2, 5, and 8 days of incubation and frozen at −70 °C. Oxidation of samples was then analyzed by SDS-PAGE as usual except that the βME was eliminated from the sample buffer. For cross-linking using the homobifunctional, cysteine-specific reagent bismaleimidohexahydroximine (BHM), the cross-linker was dissolved in Me3SO and added to in vitro translated TR to a final concentration of 0.5 mM in 25 mM HEPES (pH 7.3), 200 mM NaCl, 0.5 mM EDTA, and 20% glycerol. The reaction was performed at room temperature with constant shaking for 1 h and terminated by addition of DTT to 5 mM. Oxidation of in vitro translated TR was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 10 mM DTT. To obtain maximum cross-linking at each protein concentration, BS3 concentration was varied from 0.1 to 20 mM. The maximum amount of cross-linked dimer was used for estimating the dissociation constant for the LZ dimer. For determination of helix orientation in the LZ dimer, BMH cross-linking was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described aboveexcept in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein. To perform the same reaction under denaturing condition, 6% guanidine was included in the buffer. Oxidation of LZ was performed as described above except in buffer containing 10 μM sodium phosphate, pH 7.0, 150 mM NaCl, and 12 μM LZ protein.
protein was diluted to 400, 40, 4, 0.47, and 0.047 mM in volumes of 60 µl (400, 40, and 4 µl), 600 µl (0.47 mM), and 6 ml (0.047 mM). After cross-linking, the samples were precipitated with one-third volume of 50% trichloroacetic acid containing sodium deoxycholate (2 mg/ml), followed by boiling in SDS sample buffer and analysis by SDS-PAGE.

**Determination of Dissociation Constants**—For a single protein species, the monomer-dimer equilibrium is described by Equation 1.

\[ K_d = m^2/d \]  

(Eq. 1)

\( K_d \) is the dissociation constant, \( m \) is the concentration of monomer, and \( d \) is the concentration of dimer. To transform this equation into one that contains terms for total protein concentration (\( T \) and \( F \)) as dimer (\( F \)), let

\[ T = m + 2d \]  

(Eq. 2)

\[ F = (2d)/T. \]  

(Eq. 3)

Solving for \( m \) and \( d \) in Equations 2 and 3 and substituting these values in Equation 1 yields Equation 4.

\[ K_d = (1/F)(4T)(1 - F)^2 \]  

(Eq. 4)

Equation 4, when solved for \( F \), yields Equation 5.

\[ F = (1/4T^2)(4T + K_d - [(4T + K_d)^2 - 16T^2]^{1/2}) \]  

(Eq. 5)

Because the dimer is detected by partial cross-linking, let \( f \) be the fraction of cross-linked dimer relative to the total amount of protein and \( B \) be the ratio between \( F \) and \( f \). Thus,

\[ f = F/B \]  

(Eq. 6)

or

\[ F = Bf. \]  

(Eq. 6b)

Substituting \( f \) for \( F \) in Equation 5 yields Equation 7.

\[ f = (1/B)(1/4T^2)(4T + K_d - [(4T + K_d)^2 - 16T^2]^{1/2}) \]  

(Eq. 7)

The experimental results yield a set of \( f \) values determined by partial cross-linking at different values of \( T \), as described in the preceding section. The constant components of Equation 7, \( B \) and \( K_d \), are then determined by fitting the set of \( T \) and \( f \) pairs into Equation 7 by nonlinear regression using the curve-fitting feature of SigmaPlot (Jandel Scientific). The standard equilibrium curve is generated following Equation 5 by entering the values of \( K_d \) determined in this way. To determine how well the data points fit this curve, the (\( f \) versus \( T \)) following Equation 6b is replotted as \( f \) versus \( T \).

**Limited Proteolysis**—Samples (20 µl) containing bacterially produced, purified LZ-TR protein (20 µg) were incubated with 0, 1, 10, or 100 µg/ml proteinase K at 37 °C for 10 min. The digestions were terminated by addition of phenylmethylsulfonyl fluoride to 1 mM, and the digests were analyzed by SDS-PAGE (12%, Tris-Tricine) and stained with Coomassie Blue.

**Circular Dichroism Spectropolarimetry**—Circular dichroic spectra of the TR-LZ, LZ, and TR proteins were acquired using a JASCO J-710 spectropolarimeter calibrated with \( d_{280} \text{c} \)-camphorsulfonic acid. A water-jacketed, cylindrical quartz cuvette with a 0.05-cm path length was used. All samples were dissolved in PBS (10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl), and the spectra reported are corrected for a buffer blank. Protein concentrations were calculated as described above, and helix contents estimated from [\( \theta_{222} \)] as described by Greenfield and Fasman (21). Other methods of evaluation gave qualitatively similar results. Each spectrum represents the mean of four to eight independent scans. Samples were equilibrated at the indicated temperatures for at least 10 min prior to collecting data, and the first and last spectra at each temperature were compared to verify that the samples had attained conformational equilibrium.

**Matrix-assisted Laser Desorption-Ionization Mass Spectrometry**—The molecular masses of intact and protease-digested TR-LZ proteins were determined by matrix-assisted laser desorption-ionization (MALDI) mass spectrometry. These analyses were performed on 100–100 ng of bacterially expressed and purified TR-LZ protein using a MALDI mass spectrometer. These analyses were performed on 10–100 ng of bacterially expressed and purified TR-LZ protein using a MALDI mass spectrometer. These analyses were performed on 10–100 ng of bacterially expressed and purified TR-LZ protein using a MALDI mass spectrometer. These analyses were performed on 10–100 ng of bacterially expressed and purified TR-LZ protein using a MALDI mass spectrometer.
We find that at 25 °C about 20% of the molecules exist as dimers at a total protein concentration of 4 μM and that the fraction of dimers rises to 77% as the protein concentration increases to 123 μM (data not shown). These numbers probably represent an overestimation of dimers due to the likely effect of cross-linking on the monomer-dimer equilibrium. However, the data show that the LZ motif is capable of self-dimerization and that the fraction of dimers is dependent on the protein concentration within this range. Therefore, we estimate the lower limit of the dissociation constant ($K_d$) to be approximately 17 μM.

At 123 μM, the circular dichroism (CD) spectra of the LZ protein show that it contains a high fraction of α-helix and no β-sheet (Fig. 2A). The helical content is calculated to be 50–55% at 25 °C, which agrees well with the fact that only 77% of the molecules exist in the dimer form and that only 47 residues in the 65-residue protein are derived from the LZ motif (72%). Thus, the data are consistent with the notion that only the dimer is α-helical. CD spectra obtained at different temperatures show a cooperative and reversible loss of helicity in response to increases in temperature (Fig. 2A), confirming that the formation of the LZ helix follows two-state kinetics.

The LZ protein contains a single engineered cysteine at the N terminus of the LZ motif. To determine the relative orientation of the two LZ α-helices in the dimer, the protein was either treated with the homobifunctional, sulfhydryl-specific cross-linker BMH or allowed to auto-oxidize by air exposure. Covalent linkages formed through the cysteines may occur only if the two helices are oriented in parallel in the dimer (29). As shown in Fig. 2B, BMH cross-links the LZ protein, but does so only in the absence of guanidine denaturation, suggesting that the cross-linking is facilitated by the α-helical structure. Autooxidation also results in a covalent dimer linked by a disulfide bridge (Fig. 2B). Both results suggest that the helices are in parallel. Based on these data, we conclude that the LZ motif self-dimerizes in the form of two parallel α-helices.

Equilibrium Constants of Dimerization—A previous study using chemical cross-linking readily detected dimers of in vitro translated Ski proteins containing only the TR region of the dimerization domain (8). The failure to detect TR dimers hydrodynamically (Table I) probably reflects the fact that with the limited quantities of proteins produced by in vitro translation, the dimer exists only transiently. In contrast, under similar conditions, dimers of in vitro translated TR-LZ protein are detected hydrodynamically, suggesting that the presence of the LZ motif significantly stabilizes the dimer. To quantify the differences between the potential of these proteins to dimerize, we undertook a comparison of their equilibrium constants. For these studies, histidine-tagged TR-LZ and TR proteins were produced in bacteria and purified. These proteins were then serially diluted to desired concentrations, and the relative amounts of monomer and dimer were measured as a function of the total protein concentration. To extend the sensitivity of the measurements to the submicromolar range, a trace amount of 35S-labeled, in vitro translated protein was added as a tracer.

After reaching equilibrium during a 16-h incubation, the samples were treated with BS3, permitting subsequent separation and quantitation of dimer and monomer by SDS-PAGE and PhosphorImager analysis.

A general concern for using a chemical cross-linker in such analyses is that it may shift the equilibrium in favor of dimer. To minimize this potential interference, we have designed a method in which the cross-linking reaction is performed as a short pulse, during which a fixed fraction of the dimer population is cross-linked. To accomplish this, the reaction is performed in the presence of a glycine quencher. Theoretically, for glycine to be effective it should satisfy two criteria: 1) it should rapidly quench BS3 reactivity, thereby minimizing the potential equilibrium shift; and 2) it should render the efficiency of protein cross-linking, defined as the percentage of dimers cross-linked, constant over the range of protein concentrations employed. Both of these requirements should be met by employing glycine in such a vast excess over the protein that the amount of BS3-reacting lysine residues in the target protein is negligible.

To establish the conditions for this method, we first determined the range of glycine concentrations required to quench the reaction and the speed with which this is accomplished. As shown in Fig. 3A, when glycine is added to samples of TR-LZ dimer along with the cross-linker, the fraction of dimer cross-linked decreases almost linearly with glycine concentration, reaching about 50% at 100 mM glycine. Furthermore, glycine rapidly quenches the cross-linking of TR-LZ, as no dimers are detected when BS3 is preincubated with 100 mM glycine for as little as 1 min before the addition of the protein (Fig. 3B). The final test of the validity of this method was to determine whether the efficiency of cross-linking is independent of protein concentration. To accomplish this, TR-LZ was reacted with BS3 in the presence of 100 mM glycine over a protein concentration range of 1–100 μM, where TR-LZ exists exclusively as a dimer. As demonstrated in Fig. 3C, dimers of TR-LZ are cross-linked at the same 30% level over the entire range of protein concentration.

Therefore, the use of glycine at 100 mM satisfies the requirements of our analysis: it allows cross-linking of a large enough fraction of dimer (30–50%) to obtain statistically significant results, it stops the reaction rapidly, and it provides cross-linking efficiency that is independent of total protein concentration and of the volume of the reaction.

Fig. 4A shows an example of the data obtained using this quenched cross-linking method to examine the concentration dependence of TR dimer formation. In this case, the fraction of

![Fig. 2. Structure of the LZ motif.](image)
Concentrations where the deviation should be most noticeable, caused an equilibrium shift, it would result in overestimation but still fall closely to the curve. If the cross-linking assay range below saturation, the relative amounts of the TR dimer for monomer-dimer equilibrium. In the protein concentration equilibrium, we compared these data with the ideal curve performed on the TR-LZ protein. Data from duplicate experiments are plotted as a function of total protein concentration and quantified by PhosphorImager analysis as described under "Materials and Methods." The cross-linked fraction is plotted as a function of glycine concentration (solid line), but in the range of 1–100 mM it is nearly linear (dotted line). B, to determine the speed of glycine quenching, samples of BS3 (2 mM) were preincubated with 100 mM glycine for various periods of time before addition of 10 mM TR-LZ. The fraction of dimers cross-linked was then analyzed by Tris-Tricine SDS-PAGE and quantified by PhosphorImager analysis as described under "Materials and Methods." The cross-linked fraction is plotted as a function of glycine concentration. The effect of glycine concentration is quadratic, and quantified by PhosphorImager analysis as described under "Materials and Methods." The cross-linked fraction is plotted as a function of glycine concentration. The fraction of dimer at each concentration is derived from these data. C, TR-LZ at 1 to 100 mM was mixed with 100 mM glycine and reacted with 2 mM BS3. The cross-linked dimer fraction was determined as described above and is plotted as a function of TR-LZ concentration.

**FIG. 3.** Protein cross-linking in the presence of excess glycine. Chemical cross-linking of bacterially produced pure TR-LZ protein was analyzed as a function of glycine concentration (A), time of glycine pretreatment (B), and protein dilution (C). For quantitation, a small amount of 35S-labeled, in vitro translated TR-LZ was mixed with the bacterially produced protein as a tracer. A, samples containing 50 μM TR-LZ (completely dimeric at concentrations above 1 μM) were mixed with increasing amounts of glycine and then cross-linked with 2 mM BS3. Monomer and cross-linked dimer were separated by SDS-PAGE and quantified by PhosphorImager analysis as described under "Materials and Methods." The cross-linked fraction is plotted as a function of glycine concentration. The effect of glycine concentration is quadratic but in the range of 1–100 mM it is nearly linear. B, to determine the speed of glycine quenching, samples of BS3 (2 mM) were preincubated with 100 mM glycine for various periods of time before addition of 10 mM TR-LZ. The fraction of dimers cross-linked was then analyzed by Tris-Tricine SDS-PAGE (10%). Lane 1, 14C-labeled protein standards; lane 2, 10 μM TR-LZ reacted with 2 mM BS3; lanes 3–7, TR-LZ reacted with 2 mM BS3 preincubated with glycine for 1, 2, 4, 8, and 16 min, respectively; lane 8, 10 μM TR-LZ without cross-linking. C, TR-LZ at 1 to 100 mM was mixed with 100 mM glycine and reacted with 2 mM BS3. The cross-linked dimer fraction was determined as described above and is plotted as a function of TR-LZ concentration.

TR cross-linked increases as the total protein concentration is raised from 0.3 μM to 300 μM. Similar experiments were also performed on the TR-LZ protein. Data from duplicate experiments are plotted as a function of total protein concentration (Fig. 4B). To assess the possible effect of cross-linking on reaction equilibrium, we compared these data with the ideal curve for monomer-dimer equilibrium. In the protein concentration range below saturation, the relative amounts of the TR dimer determined experimentally fall almost exactly on the ideal curve, whereas those of the TR-LZ dimers are slightly scattered but still fall closely to the curve. If the cross-linking assay caused an equilibrium shift, it would result in overestimation of the dimer and consequently a skewed distribution of the data points. Because this is not found, especially at lower protein concentrations where the deviation should be most noticeable, such an equilibrium shift must be either completely prevented by the quick quenching method or reduced to an insignificant degree. The equilibrium constants (Kd) derived from these data are 4 × 10⁻⁶ M for TR and 2 × 10⁻⁹ M for TR-LZ. The large difference in the dissociation constants is entirely due to the leucine zipper motif, which is present in TR-LZ but lacking in TR. Thus, the data suggest that the leucine zipper motif cooperates with the tandem repeat motif in forming the TR-LZ dimer.

**Circular Dichroism Analysis of the TR and TR-LZ Dimers—** Both the TR and TR-LZ proteins are predicted to be highly α-helical; the Chou-Fasman (22), GOR (23), and PHD (24) algorithms predict that each contains between 70% and 80% helix. To determine whether the dimers are in fact α-helical, we have analyzed their secondary structures by circular dichroism spectropolarimetry. As depicted in Fig. 5, at concentrations at least 30-fold greater than the dimerization equilibrium constants calculated above (panels C and D), the spectra of both proteins are consistent with the presence of a significant proportion of α-helix.

In isolated protein domains capable of forming α-helical coiled-coils, or in smaller model peptides capable of interacting in solution (25–27), helix content is frequently observed to increase with protein concentration, reflecting stabilization of the individual helices upon their dimerization. To assess this possibility with the Ski domains, we examined their CD spectra as a function of protein concentration over the range 5–150 μM for TR and 4.8–44 μM for TR-LZ (dimer Kd = 4 and 0.02 μM, respectively). We are unable to extend the concentration range to values below the dimerization Kd for TR-LZ due to insufﬁcient dichroic signal, and consequently the ϵ222 values for this protein display no evidence for concentration-dependent helix formation (data not shown). In contrast, the helix content of TR is clearly concentration-dependent, varying from about 70% at
Two-stranded Parallel Helices in Ski

Fig. 5. CD analysis of the TR and TR-LZ dimers. A–C, effects of protein concentration and temperature on the CD spectra of TR. CD spectra of TR were recorded in PBS as described under “Materials and Methods” and represent the means of four independent scans for each curve. The spectra were recorded after samples were equilibrated at 10, 16, 20, 25, 30, 35, 40, 45, and 50 °C for 5 μM TR (A); at 13, 20, 25, 31, 37, 44, 50, 60, and 65 °C for 27 μM TR (B); and at 23, 28, 33, 38, 44, 50, 55, 60, and 70 °C for 150 μM TR (C). D, effect of temperature on the CD spectra of TR-LZ. CD spectra of 44 μM TR-LZ were recorded as described above at 23, 28, 33, 38, 44, 48, 54, 60, and 70 °C, with the first two temperatures yielding the identical spectra. E, thermal stability of the TR and TR-LZ helices. Residual helicities at the indicated temperatures are defined as the remaining helix content at a particular temperature relative to the starting helix content obtained at the lowest temperature and are expressed in percent. The values were obtained from the data depicted in A–D and from parallel experiments carried out at the indicated protein concentrations. ○, □, and △ indicate melting curves for TR at 5, 27, and 150 μM, respectively. ⧫ and ▽ indicate melting curves for TR-LZ at 4 and 44 μM, respectively.

Fig. 6. Limited proteolysis of the TR-LZ dimer. Bacterially produced, pure TR-LZ at 20 μM was cleaved with increasing amounts of proteinase K, and the digests were separated by SDS-PAGE (12%) and stained with Coomassie Blue. Lanes 1 and 2, protein standards; lane 3, blank sample containing only proteinase K at 100 μg/ml; lanes 4–7, TR-LZ digested with 0, 1, 10, and 100 μg/ml proteinase K.

27–150 μM to and a temperature of 10–13 °C to a significantly lower level at 5 μM (Fig. 5, A–C). Because the spectral data are too noisy at 5 μM to accurately apply a deconvolution algorithm, we cannot estimate the helix content at this low concentration. However, it is clear from the data that the helix content of TR at this concentration is substantially less than at the higher concentrations.

We next asked whether the thermal stability of the observed secondary structural elements differs across this range of protein concentrations by measuring the circular dichroism spectra of both proteins at different concentrations and between 10 ° and 70 °C. The resulting reduction in helicity shown in Fig. 5E is at least 90% reversible upon cooling to lower temperatures (data not shown), and the existence of an isodichroic point at about 203 nm is indicative of a simple two-state process. The results demonstrate that both proteins lose most of their secondary structure over this temperature range, but that overall the helical content of TR-LZ is more stable than that of TR. As anticipated from the fact that both of the TR-LZ spectra were recorded at concentrations significantly greater than K_d, the melting curves display no concentration dependence. We cannot make a definitive statement on the effect of protein concentration on the helicity of TR-LZ because we are unable to perform the analyses at the nanomolar protein concentrations required. In contrast, the thermal stability of TR protein is clearly concentration-dependent, such that loss of helicity occurs to a far greater extent at 5.4 μM than at 27 μM or 150 μM. This concentration-dependent stability is consistent with the existence in the dimer of interfacial contacts between two α-helical TR polypeptides. Because the entire TR sequence represents a subset of TR-LZ, we predict the existence of the same helical structure in the larger protein as well.

Limited Proteolysis—To provide independent validation of the two-stranded α-helical model for Ski dimerization, the quaternary structure of the TR-LZ dimer was probed by limited proteolysis. Assuming the α-helical structure is more resistant to hydrolysis than interconnecting loops, the fragmentation pattern would indicate whether the helices in the TR-LZ dimer are continuous. Pure TR-LZ protein (at 20 μM concentration to ensure complete dimerization) was partially digested with proteinase K, a nonspecific protease, and the products analyzed by SDS-PAGE. The predicted molecular weight of the full length histidine-tagged TR-LZ protein is 25.3 kDa, although its apparent size is 28 kDa in SDS-polyacrylamide gels (Fig. 6, lane 4). Analysis of the digests shows that the majority of the full size protein is converted from 28 kDa to a 26.4-kDa interme-
diate and then to a 23.7-kDa core (Fig. 6, lanes 5–7). The expressed TR-LZ protein contains a 25-residue histidine tag (2.6 kDa) at the N terminus and a 16-residue highly polar segment (1.7 kDa) at the C terminus (Fig. 1), neither of which is predicted to be helical. The two-step conversion of the full-size protein into the 23.7-kDa core is thus consistent with sequential cleavages of these C-terminal and N-terminal extensions, leaving a polypeptide of sufficient size to include both the tandem repeat and the leucine zipper motifs.

Protease treatment of TR-LZ also produces minor products of 14.7 kDa and 9.0 kDa and an accumulation of fragments smaller than 6 kDa upon heavier digestion (Fig. 6, lanes 5 and 6). The 14.7-kDa and 9.0-kDa fragments are apparently converted from the 23.7-kDa fragment and are close to the predicted sizes of the TR and LZ domains, respectively. This suggests a possible break in the helical structure between these regions. In fact, the TR-LZ sequence contains only one predicted loop, which is located at the junction of the TR and the LZ motifs (Asp\(^{673}\)-Cys\(^{676}\)) (Fig. 1). However, even when these bands are most apparent, the vast majority of the TR-LZ protein is found in the 23.7-kDa protease-resistant core, strongly suggesting that any break in the helices must be very short, and that the tandem repeat and leucine motifs form a nearly continuous helix in the dimer.

Because the apparent mass of the TR-LZ protein on SDS-PAGE does not agree with its predicted mass, actual sizes of proteolytic products could only be estimated. To obtain more accurate estimates, molecular masses of intact and protease-digested TR-LZ proteins were determined by MALDI mass spectrometry. The results (Fig. 6, under MALDI) show that the undigested TR-LZ has the predicted molecular mass of 25.3 kDa when standardized with carbonic anhydrase (28,987 Da).

As might be expected when analyzing the somewhat heterogeneous proteinase K cleavage products, the MALDI peaks are broadened (data not shown), but there are major peaks centering at masses of 23.0 kDa and 21.5 kDa, which agrees well with the loss of the N terminus and both termini, respectively. The spectra also contain minor species of 16.3 and 14.0 kDa consistent with the mass of the TR region after partial cleavage at the predicted loop region alone and in combination with cleavage of the N terminus, respectively. These results are consistent with our interpretation of the SDS-PAGE analysis of protease K-digested TR-LZ.

**Interchain Disulfide Bond(s) Formation**—The results presented above indicate that TR dimerize as helices, but the orientation of the two helices with respect to one another has not been addressed. To explore this question, we have taken advantage of the ability to cross-link TR with BS\(_3\), which cross-links primary amino groups, produces a cross-linked dimer band, which migrates slightly faster (Fig. 7B, left panel). Immediately after translation, TR migrates as a single band of apparent molecular mass of 18.5-kDa monomer on a non-reducing SDS-polyacrylamide gel (lane 2). However, when the protein is exposed to air at 4 °C for 1 day, a doublet band of about twice the mass of the monomer (38 kDa) is detectable (lane 3). With longer periods of air exposure, the relative intensity of the 38-kDa band increases at the expense of the 18.5-kDa monomer band (lanes 4–6). The 38-kDa species is shown to result from covalent cross-linking by disulfide bond(s) formation because the linkage is promoted by air oxidation and reversed by treatment with the reducing agent βME (lane 7).

Moreover, chemical cross-linking of TR with BMH, a cysteine-specific reagent, produces a cross-linked doublet of the same SDS-polyacrylamide gel mobility as the oxidation-induced dimer, whereas the BS\(_3\), which cross-links primary amino groups, produces a cross-linked dimer band, which migrates slightly faster (Fig. 7B, right panel). Either one or both of the cysteine residues may participate in formation of the linkage, which is likely the reason for the disulfide-linked species forming a doublet. However, regardless of which residue is involved, the ability to generate a disulfide link(s) between these residues shows that the two helices in the TR dimer are oriented in parallel.

We have taken advantage of the ability to cross-link TR dimers by auto-oxidation to obtain hydrodynamic data on this otherwise unstable species. The result obtained using the disulfide-linked TR confirm that it is a dimer (Table I). Its axial ratio of 7:1 is consistent with an overall shape of a two-stranded parallel helix.

Considering how readily the disulfide cross-links form in vitro, we have investigated the possibility that native intracellular c-Ski protein may contain a disulfide linkage between the same set of cysteines. Neither endogenous nor virally overexpressed c-Ski was found to contain such a linkage by Western
that the equilibrium favors dimer formation at higher salt concentrations. Cross-linking increases with NaCl concentration, suggesting that the dimer is more stable at higher salt concentration. The effect of salt diminishes as the NaCl concentration in reaction buffer is reduced. The stability of the GCN4 leucine zipper decreases with increasing salt concentration up to 0.5 M, suggesting that electrostatic interactions contribute negatively to the dimerization affinity. We therefore tested the effect of NaCl concentration on dimerization. Using BS³ in the presence of 200 mM (○), 50 mM (▲), or 5 mM (□) NaCl, cross-linked dimer was separated by SDS-PAGE and quantified relative to the total amount of TR using the PhosphorImager as described under "Materials and Methods." The relative amount of cross-linked dimer is plotted as a function of BS³ dosage. The effect of salt concentration is shown by the shift of the cross-linking curve to the lower right as the NaCl concentration in reaction buffer is reduced.

**Effect of NaCl Concentration on TR Dimerization—Dimerization in vitro**

Protein TR was translated in vitro with [³⁵S] label and reacted with various amounts of BS³ in the presence of 200 mM (○), 50 mM (▲), or 5 mM (□) NaCl. Cross-linked dimer was separated by SDS-PAGE and quantified relative to the total amount of TR using the PhosphorImager as described under "Materials and Methods." The relative amount of cross-linked dimer is plotted as a function of BS³ dosage. The effect of salt concentration is shown by the shift of the cross-linking curve to the lower right as the NaCl concentration in reaction buffer is reduced.

**DISCUSSION**

In the present work, we have examined the structure of the C-terminal dimerization domain of c-Ski and the relative roles of its two subdomains in dimer formation. Our hydrodynamic, cross-linking, and circular dichroism data all suggest that the domain does in fact form a helical structure as proposed in earlier studies (8, 10). The axial ratios of the TR and TR-LZ dimers are 7:1 and 10:1, respectively, suggesting a rodlike overall shape that is characteristic of two-stranded helices. Furthermore, the CD spectra indicate that these dimers contain almost exclusively α-helical structure. The existence of a tight structure that is formed of the TR and LZ helices is also supported by the limited proteolysis of the TR-LZ dimer, which reveals a relatively protease-resistant core consisting of both the TR and LZ motifs. Finally, a direct relationship between dimerization and helicity is observed, suggesting that the association occurs through a helical interface (25–27). Thus, the stability of TR helices is clearly a function of both temperature and concentration. The fact that the TR-LZ dimers dissociate only at protein concentrations below the sensitivity of our measurements precludes our being able to observe this process spectrally. Nonetheless, the higher dimerization affinity of TR-LZ as compared with TR alone correlates with a higher helical content and helix stability.

Both the TR and LZ dimers are formed of two parallel helices, as shown by the formation of the terminal disulfide bridges upon air oxidation. The fact that the two are subsets of TR-LZ suggests that the TR-LZ dimer also contains parallel helices. This arrangement is also in agreement with the observation that the TR-LZ dimer displays a greater axial ratio than does the TR dimer, and that no large loops are detected by limited proteolysis in the TR-LZ dimers as would be required for accommodating anti-parallel helices. The TR and LZ helices rather appear to be connected by a small loop that is partially shielded from proteolysis. The sizes of proteolytic fragments place this loop at the end of the fifth tandem repeat, within a short segment predicted to be a loop by the PHD program (24).

The functional roles of the TR and the LZ motifs are clarified in this study. Expressed independently, neither TR or LZ self-dimerizes efficiently. Linked together as one protein, the two motifs produce a cooperative effect on the dimerization affinity. This suggests that the α-helical structure consists of both motifs whose interaction is greatly enhanced by the extension of the hydrophobic interface. The K₆₇ of the TR-LZ dimer approaches 2 × 10⁻⁸ M, reflecting a dimerization affinity 2 orders of magnitude higher than that of the TR dimer alone. The levels of endogenous Ski are extremely low (1, 33), and such a high affinity may be needed to assure dimerization. It has been suggested that the biological activity of Ski depends upon its ability to dimerize, and that the lack of the TR-LZ domain in v-Ski is responsible for its decreased transforming potency as compared with c-Ski (8).

We have attempted to construct a dimer model for the TR-LZ domain using a helical net representation of its amino acid sequence and the canonical coiled-coil structure as represented by the GCN4 leucine zipper (29). The LZ subdomain fits this model well and, like GCN4, the two parallel LZ helices cross over at an angle of 18° to form a continuous, mostly hydrophobic interface involving heptad positions 1 and 4 (Fig. 9A). This is not the case for the TR subdomain. No continuous hydrophobic streak can be found on its helical surface. In fact, docking the two TR helices at the 18° crossover angle, as in the GCN4 coiled coil, causes extensive clashes between residues of the same charge. We therefore systematically changed the crossover angle until we found one which maximized hydrophobic interactions, particularly those involving the core element.
Two-stranded Parallel Helices in Ski

Fig. 9. Models for Ski dimerization. A, a helical net diagram (34) of the LZ domain of Ski (Fig. 1) with residue spacing and 18° crossing angle taken from the structure of GCN4 by O'Shea et al. (29). Residues 657–733 are indicated by the single-letter notation and are arrayed bottom to top in N- to C-terminal orientation. Shaded circles are residue positions on one monomer and open circles on the facing monomer of the dimer. The “zipper” residues at heptad positions a and d that form the dimer interface are indicated by bold letters and circles. Note the balance of basic and acidic residues at the positions (e and g) adjacent to the zipper. B, helical net diagram of the dimer formed by the three C-terminal repeats (residues 603–671) of the TR subdomain of Ski (Fig. 1) with residue spacing as in A but a crossing angle of 4°. The core residues of the TR (LXXELEXLR) are indicated by bold circles and letters. Note how these residues interdigitate to form hydrophobic patches on the helical interface and balanced charge at the border of the interface. C, schematic of a dimer formed by the entire TR-LZ domain of Ski. The TR and LZ subdomains are separated by a short, protease-cleavable loop, which contains the two cross-linkable cysteine residues and accommodates the differences in crossing angles of the two subdomains. The sizes of the subdomains conform to the results of partial proteolytic digestion.

(LXXELEXLR) of the tandem repeat (Fig. 1). Instead of a continuous hydrophobic zipper, we find hydrophobic “buttons” (Fig. 9B) formed by the leucine residues of the core element when we use a 4° crossover angle (Fig. 9B). These “leucine buttons” (three of which are shown in Fig. 9B) produce five discontinuous hydrophobic interfacial contacts separated by regions rich in lysine, arginine, and polar residues. Docking of two parallel TR helices at an interhelical crossover angle of 4° would then occur in a “button up” fashion by pairing the five hydrophobic pockets on each of the helices to produce a dimer (Fig. 9B). This arrangement also pairs most of the arginine and glutamate residues of the core motif at the positions adjacent to the interfacial contacts. This would allow ionic interactions, which could contribute a stabilizing effect as has been proposed for the leucine zipper coiled coil (29).

Our model for the TR-LZ dimer thus contains two subsets of helices: the TR helices, which are “buttoned” together by a discontinuous hydrophobic interface at a 4° crossover angle, and the LZ coiled coil held together by a continuous leucine zipper with an 18° crossover angle (Fig. 9C). The difference in the interhelical crossover angle is accommodated by the small connecting loop that is indicated by the results of partial proteolytic digestion. The residues involved in dimerization of the TR helices have not yet been defined by either mutagenesis or structure determination, but our current data show that dimerization of TR is stimulated by increases in salt concentration, suggesting that the process is driven by hydrophobic interactions. Furthermore, the LXXELEXLR motif is the most conserved element of the repeated sequences in Ski, and it is also repeated in the C-terminal region of SnoN that is involved in heterodimerization with Ski (8, 10). The conservation of this sequence element in an otherwise highly diverged region of the two proteins suggests that these residues are involved in homodimerization of Ski and heterodimerization between Ski and Sno. Therefore, although unusual and quite speculative, the proposed structure for the TR helices is attractive because it maximizes hydrophobic interactions involving this conserved motif.

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