J. Mol. Biol. (1996) **256**, 829–837

JMB

Regulatory Region C of the *E. coli* Heat Shock Transcription Factor, σ^{32}, Constitutes a DnaK Binding Site and is Conserved Among Eubacteria

John S. McCarty[1], Stefan Rüdiger[1], Hans-Joachim Schönfeld[2] Jens Schneider-Mergener[3], Kenji Nakahigashi[4], Takashi Yura[4] and Bernd Bukau[1]*

[1]*Zentrum für Molekulare Biologie Heidelberg Universität Heidelberg INF 282, 69120 Heidelberg FRG*

[2]*Hoffmann-La Roche Limited Pharmaceutical Research-New Technologies, CH-4002, Basel Switzerland*

[3]*Universitätsklinikum Charité Humboldt-Universität Schumannstr. 20-21 D-10098 Berlin, FRG*

[4]*HSP Research Institute Kyoto Research Park, Kyoto 600, Japan*

**Corresponding author*

The *E. coli* heat shock response is regulated at the transcriptional level through stress-dependent controls of the heat shock promoter-specific σ^{32} subunit of RNA polymerase. A key aspect of this regulation, the sensing of stress and transmission of this information to σ^{32}, involves the chaperone system formed by the DnaK, DnaJ and GrpE heat shock proteins. This system mediates stress-dependent controls of levels and activity of σ^{32} which rely, at least in part, on direct association of DnaK and DnaJ with σ^{32}. We identified DnaK binding sites within the σ^{32} sequence by probing a cellulose-bound peptide library scanning σ^{32}. Two sites with high affinity for DnaK, containing the motifs RKLFFNLR and LRNWRIVK, were located centrally and peripherally, respectively, to the region C of σ^{32}, previously implicated genetically in chaperone-dependent control of σ^{32} levels. Cloning and sequencing of *rpoH* homologs from five Gram-negative proteobacteria revealed that region C, including the DnaK binding motif central to it, is highly conserved among σ^{32} homologs but missing in other σ factors. We propose that binding of DnaK to region C is central to a conserved regulatory mechanism allowing the sensing of stress by the heat shock gene transcription machinery.

Keywords: DnaJ; Hsp70; heat shock response; sigma factor; *rpoH*

Introduction

The heat shock response of cells is generally regulated at the transcriptional level through stress-dependent positive control by the heat shock transcription factor. In *Escherichia coli*, the positive regulator is the *rpoH* gene product, the heat shock promoter-specific σ^{32} subunit of RNA polymerase (Bukau, 1993; Georgopoulos *et al*., 1994; Gross *et al*., 1990; Yura *et al*., 1993). The heat shock response is induced as a consequence of increased σ^{32} levels, resulting from increased synthesis and stability of σ^{32}, and, possibly, of stimulated σ^{32} activity. The response is shut off as a consequence of a decline in σ^{32} levels and inhibition of σ^{32} activity (Gross *et al*., 1990; Yura *et al*., 1993).

Key elements of the stress-dependent controls of σ^{32} are the DnaK (Hsp70), DnaJ (Hsp40) and GrpE heat shock proteins, which constitute a chaperone system for protein folding (Georgopoulos *et al*., 1994). Genetic evidence indicates that they act as negative modulators of the heat shock response by mediating efficient σ^{32} degradation, repression of σ^{32} activity and, during the adaptation phase, repression of σ^{32} synthesis (Grossman *et al*., 1987; Straus *et al*., 1989, 1990; Tilly *et al*., 1983; C. Gross, personal communication). The DnaK chaperone system was recently shown to physically interact with σ^{32} (Gamer *et al*., 1992, 1996; Liberek *et al*., 1992; Liberek & Georgopoulos, 1993). In this process, DnaJ activates DnaK for stable σ^{32} binding in the presence of ATP (Gamer *et al*., 1996; Liberek & Georgopoulos, 1993) and GrpE accelerates dissociation of this complex (Gamer *et al*., 1996). These interactions control the activity of σ^{32} (Gamer *et al*., 1996) and might mediate the chaperone-dependent stability and synthesis control of σ^{32}. Several regulation models propose that induction of the heat shock response relies on sequestering of the DnaK system

Abbreviations used: IAANS, 2-(4'-(iodoacetamido)-anilino)naphthalene-6-sulfonic acid; PVDF, polyvinylene difluoride.

0022–2836/96/100829–09 $18.00/0

away from σ^{32} through binding to damaged proteins accumulating during stress (Bukau, 1993; Craig & Gross, 1991; Gamer *et al*., 1996; Georgopoulos *et al*., 1994).

Identification of the DnaK and DnaJ binding site(s) within σ^{32} is central to an understanding of the signal transduction pathway converting stress to a heat shock response in *E. coli*. According to the proposed regulation models, the affinity of DnaK and DnaJ for these site(s) determines the ability of other substrates, including misfolded proteins, to sequester these chaperones and to induce the heat shock response. A genetic approach led to the identification of a regulatory region within the *rpoH* coding sequence, referred to as region C, required for the chaperone-dependent control of σ^{32} stability and synthesis of σ^{32} (Nagai *et al*., 1994). This region, spanning residues 122 to 144 of σ^{32}, possibly with some additional proximal residues, exhibits its regulatory activity at the protein level.

We report the identification of DnaK binding sites within the σ^{32} sequence by screening of a peptide library. Two high affinity binding sites are located central and peripheral to the regulatory region C of σ^{32}. Region C is found specifically conserved among σ^{32} homologs of eubacteria. We propose that the binding of DnaK to region C of σ^{32} constitutes an important element of a conserved regulatory mechanism connecting the cellular stress status to the heat shock transcription machinery.

Results

Identification of DnaK binding sites within σ^{32}

We utilized a cellulose-bound peptide library scan of the σ^{32} sequence to search for DnaK binding sites. The scan contained 13mer peptides, a length significantly longer than the minimal length of seven residues reported to be required for efficient peptide binding to the BiP homolog of DnaK (Flynn *et al*., 1991). Starting with a peptide comprising the N-terminal 13 residues of σ^{32}, the N termini of the following peptides move by three amino acid steps along the sequence of σ^{32} resulting in an overlapping window of ten residues. Therefore, the library gives assurance of presenting DnaK all possible linear recognition sites contained within the σ^{32} sequence. DnaK was allowed to react with the library to equilibrium, followed by elimination of unbound DnaK, electrochemical transfer of peptide-bound DnaK to polyvinylene difluoride membranes and detection of the transferred DnaK by immunoblot analysis. Screening the library by this method allowed identification of at least seven major, potential DnaK binding regions within the σ^{32} sequence (Figure 1(a)).

Experiments were performed to assure that this screen yielded valid information on DnaK binding sites in σ^{32}. First, inclusion of a competitor substrate for DnaK binding, peptide C of vesicular stomatitis virus glycoprotein (Flynn *et al*., 1989), in solution during incubation of DnaK with the membrane resulted in reduced DnaK binding to the cellulose-bound peptides (data not shown). This indicates that the recognition of peptides on the membrane is specific and occurs *via* the substrate binding site of DnaK. Second, we confirmed for peptides from major binding regions (Figure 1(a)) that they bind DnaK with significant affinity also in solution (Tables 1 and 2; see below for experimental approaches). Peptides from two sites, Q132 to Q144 and M195 to N207 (spots 44 and 65), had the highest affinities for DnaK (K_D values of 10^{-7} M), while peptides from the other sites had lower affinities (K_D values between 1 and 7 μM). In addition, two peptides (spots 13 and 47) with very little or no detectable DnaK binding in the membrane assay also failed to show significant binding in solution ($K_D > 10$ μM). Thus, the cellulose-bound peptides and the corresponding peptides in solution exhibit qualitatively similar DnaK binding properties, although quantitative differences in the apparent affinities exist for some peptides. We conclude that the membrane library screening approach allows the identification of DnaK binding sites within the σ^{32} sequence.

Each of the seven identified DnaK binding regions might *a priori* be relevant for regulation of σ^{32}. DnaK has the ability to bind non-native polypeptides through interaction with hydrophobic segments which usually are buried inside the core of the native protein. Most of the identified DnaK binding sites might therefore be in the core of σ^{32} and not be available for DnaK binding to the folded σ^{32}. For the two DnaK binding sites located within residues L118 to K125 and R133 to R140 (Figure 1(b)), however, genetic evidence exists for a regulatory role. These sites are located peripheral and central, respectively, to the region C centered within residues 122 to 144 of σ^{32} which has been genetically implicated in the DnaK, DnaJ, and GrpE-mediated stress control of synthesis and stability of σ^{32} (Nagai *et al*., 1994). This genetic evidence together with our biochemical evidence for DnaK binding sites within and close to region C, led us to characterize the chaperone binding properties of region C and neighboring residues in more detail.

Peptides from region C bind stably DnaK but not DnaJ

To further define the sequences encompassing and surrounding region C that are involved in DnaK binding, we synthesized various peptides from this region (see Table 2) and characterized their binding properties in solution. As DnaJ is also implicated in σ^{32} regulation, we tested these peptides for DnaJ binding as well. In one approach, we determined the ability of the test peptides to compete with well characterized reporter substrates which bind stably during gel filtration to DnaK (peptide C of vesicular stomatitis virus glycoprotein; Buchberger *et al*., 1994) or DnaJ (native σ^{32} protein; Gamer *et al*., 1992). By this competition test (Table 2), four overlapping

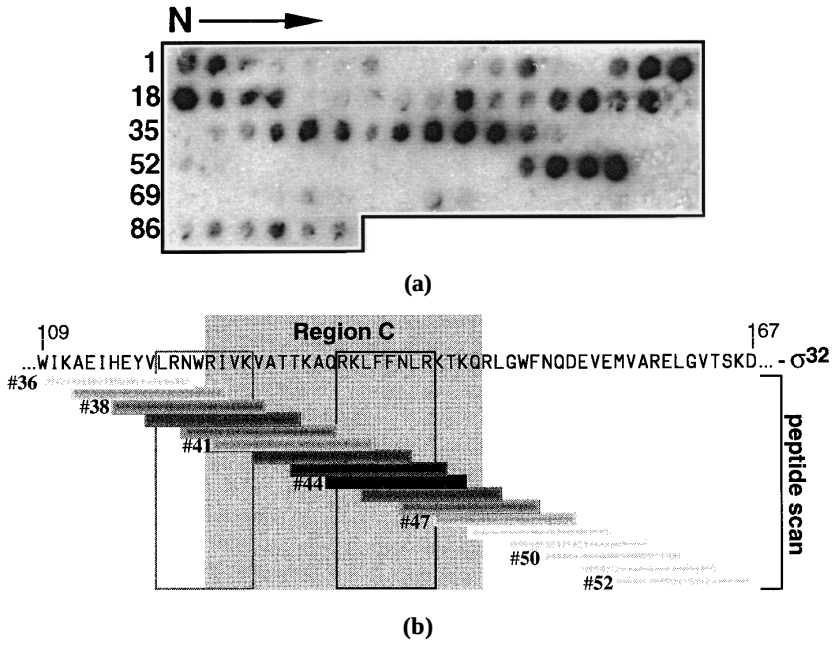


Figure 1. Binding of DnaK to a cellulose-bound peptide library scanning σ^{32}. (a) DnaK was incubated with a cellulose membrane containing a σ^{32} peptide library, followed by electrotransfer of peptide-bound DnaK onto a PVDF membrane and visualization by immunostaining. Peptides (13mers) start with the N-terminal σ^{32} sequence (upper left; labeled 1) and scan the entire σ^{32} sequence to the C terminus (lower right; labeled 91) with a three-residue step. Repetitions of this experiment using independently synthesized peptide libraries yielded identical results. The major DnaK binding regions of the σ^{32} sequence are spots 1 to 3 (residues M1 to Y19 of the σ^{32} sequence), 15 to 21 (H44 to P74), 30 to 33 (V90 to A111), 38 to 40 (H114 to Q132), 42 to 46 (V126 to N150), 63 to 66 (D189 to D210) and 87 to 91 (G261 to A285). (b) A segment of σ^{32} (from residue W109 to D167) comprising region C (gray, residues R122 to Q144) is shown with an alignment of the library peptides scanning this region. The apparent strength of DnaK binding to these peptides is judged by the relative darkness of the peptide spots (see (a)) and is symbolized by the darkness of the bars. The two proposed DnaK binding sites are shown as two boxed stretches of eight amino acids, in accordance with the reported minimal length of seven residues required for optimal peptide binding to the BiP homolog of DnaK (Flynn *et al*., 1991). They were assigned on the basis of both the presented alignment of region C peptides as well as the common features of more than 200 DnaK binding peptides identified by screening additional peptide libraries (S.R., J.M., J.S.-M. and B.B., unpublished results).

peptides that we synthesized from the region C area were identified as binding strongly to DnaK, in agreement with the peptide library analysis. In contrast, none of the peptides provided substantial competition to stable binding of DnaJ to σ^{32} protein, even when the peptides were in 100-fold molar excess of σ^{32}.

In another approach, peptide binding was determined by the fluorescent response of a fluorescent dye, 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS), conjugated to cysteine on the C termini of five peptides (Table 2). For IAANS-labeled peptides with affinity for DnaK, such as σ^{32}-Q132-Q144-C-IAANS, the addition of excess DnaK yielded substantial increases, to up to 20-fold, in fluorescence emission. We observed strong increases in the fluorescent signals of the same peptides that were identified as active in binding in the competition assay (Figure 2(a) and Table 2). The differences in absolute levels of fluorescence signals of peptides binding to DnaK might not solely reflect their affinity for DnaK since these peptides might also differ with respect to the positioning of the fluorophor within the substrate binding pocket of DnaK. In contrast to DnaK, however, no significant changes in fluorescent signals were observed when DnaJ was added to the IAANS conjugated peptides. Thus by two criteria, the inability of peptides to compete for a reporter substrate and lack of fluorescent signal upon DnaJ binding to fluorescently conjugated peptides, DnaJ does not bind to peptides derived from the sequences within or around region C. We do not rule out that for unknown reasons our assays were unable to detect an existing affinity of DnaJ for binding to region C sequences.

The σ^{32}-Q132-Q144-C-IAANS peptide was titrated against DnaK and the fluorescence response curve found to fit with a dissociation constant (K_D) of 78 nM for binding of this peptide to DnaK. Subsequently, the dissociation constants of the other, non-conjugated peptides of region C were unambiguously determined in solution (Hulme & Birdsall, 1992) by their abilities to compete with σ^{32}-Q132-Q144-C-IAANS for binding to DnaK. The K_D values for binding of five peptides containing region C sequences to DnaK were very low between 40 and 200 nM (Table 2).

Based on sequence alignments of σ^{32}-derived peptides in the library (Figure 1(b)) and in solution

Table 1. DnaK binding properties of peptides derived from the σ^{32} sequence

Spot[a]	Sequence[b]	K_D (μM)[c]
2	4-KMQSLALAPVGNL	7
12	34-EPALAKLHYHGDL	2
13	37-LAKLHYHGDLEAA	>10
16	47-LEAAKTLILSHLR	3
27	80-QGNIGLMKAVRRF	3
65	195-MAPVLYLQDKSSN	0,1
89	267-VRQLEKNAMKKLR	3

[a] Numbers correspond to the spots shown in Figure 1(a).

[b] Sequences of the tested peptides are shown. Numbers indicate the N termini of the peptides within the σ^{32} protein sequence. Further peptides tested corresponding to spots 26, 31 and 37 were insoluble in water.

[c] K_D values were determined by fluorescence assays testing the ability of peptides to compete with σ^{32}-Q132-Q144-C-IAANS for DnaK binding. The peptides from spots 16 and 27 were found to increase at high concentrations the fluorescence signal even in the absence of DnaK. In these cases, the K_D values were determined by the ability of these peptides (at one, two and fivefold molar excess) to compete with ^{3}H-labeled peptide C of vesicular stomatitis virus glycoprotein (Flynn *et al*., 1989) for DnaK binding.

(Table 2), together with a large number of other protein-derived peptides identified to bind DnaK (S.R., J.M., J.S.-M. and B.B., unpublished results), we were able to identify two sequences enriched in hydrophobic residues, RKLFFNLR and LRNWRIVK, as responsible for the high affinity binding of DnaK to the periphery and the center of region C sequences.

Region C is conserved among σ^{32} homologs

The segment of σ^{32} encompassing and surrounding region C is poorly conserved among σ factors involved in transcription of genes other than heat shock genes (Figure 3). We determined whether conservation of region C exists among σ factors involved in heat shock gene regulation. We cloned *rpoH* homologs from five Gram-negative proteobacteria (*Enterobacter cloacae*, *Serratia marcescens*, *Proteus mirabilis*, *Agrobacterium tumefaciens*, *Zymomonas mobilis*) by complementation of the temperature-sensitive growth of Δ*rpoH30::kan* cells of *E. coli* and analyzed the cloned genes by sequencing. Figure 3 shows aligned amino acid sequences encoded by the cloned genes corresponding to region C of σ^{32}, along with previously reported σ^{32} homologs from *Pseudomonas aeroginosa* and *Citrobacter freundii* (Benvenisti *et al*., 1995; Daggett Garvin & Hardies, 1989; Naczynski *et al*., 1995). The region flanking and including region C is highly conserved within all σ^{32} homologs and is clearly divergent from the corresponding regions of other σ factors such as *E. coli* σ^{70}. In particular, the DnaK binding sequence centered in region C of σ^{32}, RKLFFNLR, was almost entirely conserved within the σ^{32} homologs, with the exception of an arginine replacement of the lysine in the *A. tumefaciens* homolog. This sequence, in particular the hydrophobic residues likely to be responsible for the strong binding to DnaK, is absent in σ^{70}. The DnaK binding sequence peripheral to region C (LRNWRIVK) was conserved in the σ^{32} group as well, though some conservative exchanges existed at several positions of the corresponding *Agrobacterium tumefaciens* and *Zymomonas mobilis* sequences.

For the region C containing segment of σ^{32}, structure prediction analysis using the PhD program (Rost & Sander, 1993) identified three stretches with strong helical propensity, one helix centered on the RKLFFNLR DnaK binding sequence and two flanking helices. The sequences intervening these helices had either no strong prediction or a predicted loop structure. We noted that this series of three helices was not only predicted for σ^{32} but also for σ^{70}, which differs in sequence within the corresponding region. This conservation of predicted structure supports the sequence alignment shown, and, furthermore, stresses the importance of the actual sequence presented by region C for determining recognition by DnaK. Together, these findings demonstrate the high sequence and structural conservation of the region C including the centrally located DnaK binding site among σ^{32} homologs.

Table 2. DnaK binding properties of peptides derived from region C

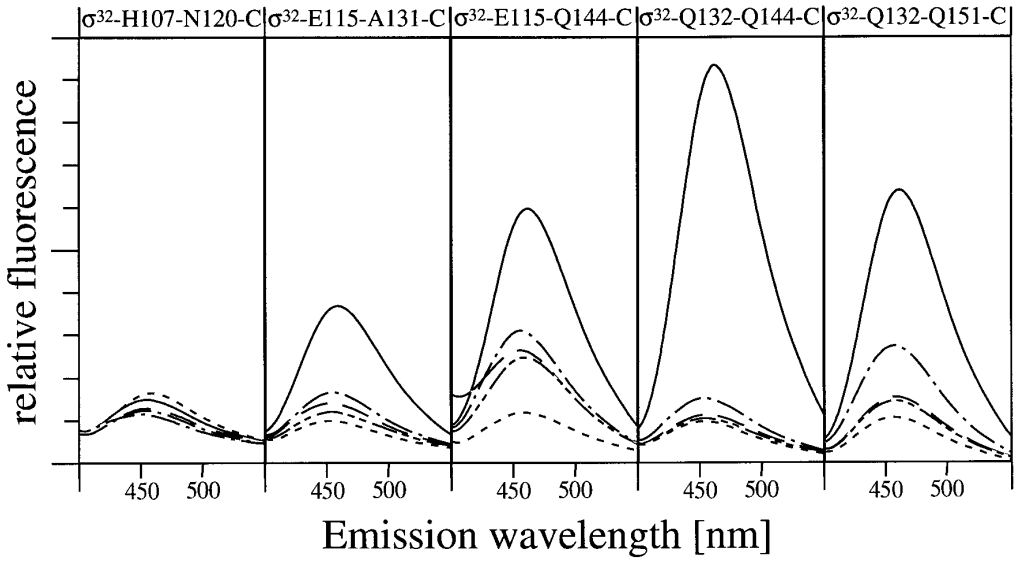
Peptide[a]	Sequence[b]	Peptide competition[c]	Flourescence increase[d]	K_D (nM)
	Region C			
σ^{32}	...HWIKAEIHEYVLRNWRIVKVATTKAQRKLFFNLRKTKQRLGWFNQDE...			
σ^{32}-H107-N120-C	HWIKAEIHEYVLRNC	–	–	$>10^4$
σ^{32}-E115-A131-C	EYVLRNWRIVKVATTKAC	+	+	200
σ^{32}-E115-Q144-C	EYVLRNWRIVKVATTKAQRKLFFNLRKTKQC	+	+	80
σ^{32}-K125-G147-KY	KVATTKAQRKLFFNLRKTKQRLGKY	n.d.	n.d.	80
σ^{32}-Q132-Q144-C	QRKLFFNLRKTKQC	+	+	100
σ^{32}Q132-Q151-C	QRKLFFNLRKTKQRLGWFNQC	+	+	40
σ^{32}-K141-E153	KTKQRLGWFNQDE	n.d.	n.d.	$>10^4$

[a] K and Y residues were present at the C terminus of σ^{32}-K125-G147-KY to increase its solubility; C residues were added to the C termini of selected peptides for IAANS conjugation.

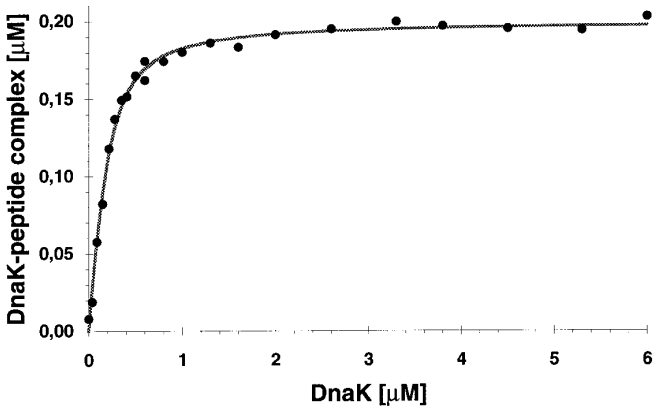
[b] The region C containing segment of σ^{32} protein (from H107 to E153) and sequences of peptides used are shown.

[c] Indicated are the ability (+) or the inability (–) of the particular peptide to compete at one or tenfold molar excess with ^{3}H-labeled peptide C of vesicular stomatitis virus glycoprotein (Flynn *et al*., 1989) for DnaK binding; n.d., not determined.

[d] Indicated are the ability (+) or the inability (–) of DnaK to increase the emission of fluorescence of the particular IAANS-conjugated peptide; n.d., not determined. The experimental data in detail are presented in Figure 2.



(a)



(b)

Figure 2. Fluorescence response of IAANS-conjugated peptides upon addition of DnaK and DnaJ. (a) Peptides (see Table 1) conjugated with IAANS through the C-terminal cysteine were incubated for 75 minutes at 30°C with no protein added (- - -) or with DnaK (continuous line), DnaJ (— —), BSA (— -) or ovalbumin (— - -), and the emission spectra were recorded with excitation at 335 nm. (b) The peptide σ^{32}-Q132-Q144-C-IAANS was titrated with DnaK and the relative fluorescence signal at 460 nm recorded as a function of the calculated free DnaK concentration. The data fit a K_D of 78 nM.

Discussion

Our analysis of the DnaK binding properties of peptides derived from σ^{32} led to the identification of seven DnaK binding regions within the σ^{32} sequence. Two regions with high affinity for DnaK are located central and peripheral to the regulatory region C of σ^{32}. We propose that binding of DnaK to region C is a key event of a chaperone-dependent, regulatory mechanism to adjust the level and, perhaps, the activity of σ^{32} to the stress status of the cell.

For identification of DnaK binding sites within the σ^{32} sequence, the screening of a library of solid phase-bound peptides scanning σ^{32} proved highly useful. This approach is based on the ability of Hsp70 chaperones including DnaK to interact with their substrates by binding to short peptide sequences (Flynn *et al*., 1991). With respect to DnaK binding, solid phase-bound peptides and the corresponding peptides in solution behaved comparably, though some differences in the relative binding affinities existed for a subset of peptides, thus validating our approach. Common to DnaK binding peptides derived from σ^{32}, including the DnaK binding peptides from region C, is a high content of hydrophobic residues. This feature is shared with peptide substrates for Hsp70 proteins including DnaK identified by panning of phage display libraries (Blond-Elguindi *et al*., 1993; Gragerov *et al*., 1994).

Two of the seven DnaK binding sites within the σ^{32} sequence detected by our peptide library screen, containing the proposed binding sequences RKLFFNLR and LRNWRIVK, are located within and peripheral, respectively, to the regulatory

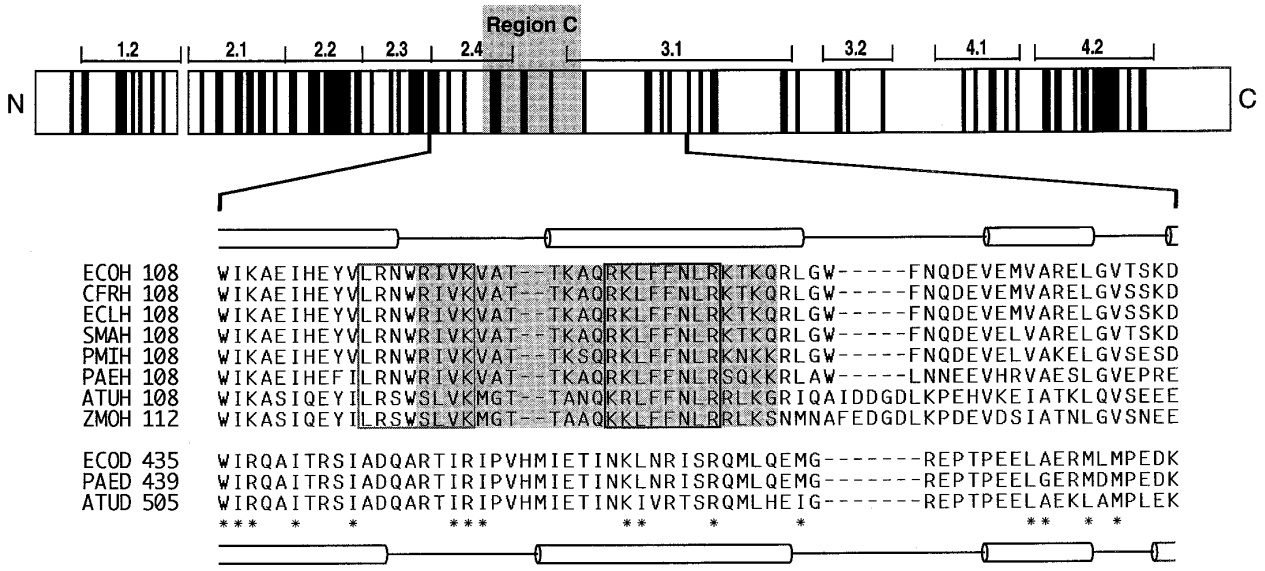


Figure 3. Sequence alignment of segments of σ^{32} homologs and unrelated σ factors corresponding to region C. The sequences of *E. coli* σ^{32} (ECOH; A94012, accession number) and σ^{32} homologs from *Citrobacter freundii* (CFRH; S04697), *Enterobacter cloacae* (ECLH; D50829), *Serratia marcescens* (SMAH; D50831), *Proteus mirabilis* (PMIH; D50830), *Pseudomonas aeroginosa* (PAEH; U09560), *Agrobacterium tumefaciens* (ATUH; D50828), *Zymomonas mobilis* (ZMOH; D50832) (upper sequence group) are aligned with the corresponding regions of σ^{70} from *E. coli* (ECOD), *Pseudomonas aeroginosa* (PAED) and *Agrobacterium tumefaciens* (ATUD) (lower sequence group). The σ^{70} and σ^{32} families have strong homology within certain regions, indicated in the upper Figure as bars and, in the lower Figure, as stars under the sequence comparison. However, there is very little homology between σ^{70} factors and the σ^{32} homologs in sequences around and including region C. In contrast to the lack of 99 homology in sequence, structure predictions using the PhD program (Rost & Sander, 1993) indicate near identity in the secondary structures of these regions between σ^{32} and σ^{70} from *E. coli*, including several α helices (tubes). The intervening regions either had no strong prediction or were predicted as loop structure and are denoted as lines. Residues considered to be conservative changes were S, T; R, K; D, E; N, Q; F, Y, W; I, L, V, M.

region C. The 23 amino acid stretch comprising region C was indicated through two pieces of genetic evidence as necessary for DnaK, DnaJ and GrpE-dependent, negative regulation of σ^{32} translation and stability (Nagai *et al*., 1994). First, σ^{32}-β-galactosidase protein fusions carrying 144 residues or more of the N terminus of σ^{32} exhibit chaperone-dependent regulation of their levels similar to authentic σ^{32}, whereas σ^{32}-β-galactosidase fusions carrying less than the 122 N-terminal amino acids of σ^{32} are defective in this regulation. Second, a σ^{32}-β-galactosidase fusion carrying most of the σ^{32} sequence (269 residues) and, in addition, an altered region C sequence (from residue 122 to 44) due to a frameshift mutation confers defects in regulation of the σ^{32}-β-galactosidase fusion protein. The region C between residues 122 and 144 therefore is essential for correct regulation of σ^{32} levels although a regulatory role of additional sequences outside this region cannot be excluded. We note that not only the short, 13mer peptides derived from region C, but also a 23mer peptide comprising the entire region C binds to DnaK with high affinity.

The high conservation of region C within functional homologs of σ^{32}, but not within other σ factors, provides further strong support for the importance of this region for heat shock-specific regulation. We propose that the chaperone-dependent regulation of σ^{32} in response to stress involves the direct binding of DnaK to region C, and that this mechanism of regulation of heat shock gene-specific σ factors is conserved within Gram-negative bacteria. The RKLFFNLR containing binding site might be the main target site for DnaK relevant for regulation as it is located entirely within region C and almost entirely conserved within σ^{32} homologs. However, it is unclear whether the strong evolutionary conservation of this sequence is driven by a need to bind to DnaK given that sequences capable of binding to DnaK show significant variability (Gragerov *et al*., 1994). It is possible that the DnaK binding site peripheral to region C of σ^{32} also plays a role in regulation, although one part (LRN) of the proposed binding motif LRNWRIVK is located outside the 23 amino acid stretch comprising region C. More complicated scenarios for chaperone-dependent regulation of σ^{32} translation and stability involving both DnaK binding sites cannot be excluded. Indeed, in initial analytical ultracentrifugation experiments, we observed the accumulation of DnaK dimers in the presence of a σ^{32} derived peptide containing both LRNWRIVK and RKLFFNLR binding sequences but not in the presence of a peptide containing only the RKLFFNLR sequence (H.-J.S. and B.B., unpublished results). This result is consistent with the interpretation that DnaK is capable, in principle, of simultaneous association with these two adjacent

binding sites. DnaJ, which binds to σ^{32} with high affinity (Gamer *et al*., 1992, 1996; Liberek & Georgopoulos, 1993) and is involved in the stability and translational regulation, was not stably bound to peptides containing sequences within or surrounding region C. The binding site for DnaJ thus remains to be elucidated.

What might be the functional consequences of DnaK binding to region C? While region C has been associated with the chaperone-dependent translation and stability control, it has not been associated with the activity control of σ^{32} though such a role is not formally excluded. Activity control necessitates chaperone binding to the folded σ^{32} (Gamer *et al*., 1996). The affinity of DnaK is lower for folded σ^{32} (K_D of approximately 5 μM) (Gamer *et al*., 1996) than for region C derived peptides (K_D approximately 100 nM). This discrepancy can be explained by assuming that in the folded σ^{32} protein the DnaK binding site in region C is in a conformation different from that in region C derived peptides. In addition, the steric surrounding of region C in the σ^{32} protein might reduce the affinity for DnaK. In any case, it remains to be determined whether the activity control of σ^{32} also involves DnaK binding to region C.

It is intriguing that region C acts at the protein level to mediate repression of translation of σ^{32} during the shut off phase of the heat shock response (Nagai *et al*., 1994), suggesting that DnaK, DnaJ and GrpE act cotranslationally. Furthermore, the chaperone and region C dependent degradation of σ^{32} occurs shortly after its synthesis as the half-life of σ^{32} is one minute or less at steady state conditions (Straus *et al*., 1987; Tomoyasu *et al*., 1995). Given these features, it seems a reasonable speculation that stability and translation control through chaperone binding to σ^{32} occurs at the nascent σ^{32} chain level. Any one of the DnaK binding regions identified by peptide library screening could *a priori* be involved in these controls. However, the genetic and biochemical evidences obtained so far are consistent with the speculation that it is region C which is presented in nascent σ^{32} in a conformation allowing high affinity binding of DnaK. The binding of DnaK to region C of the nascent σ^{32} chain, and of DnaJ to an unknown site, could then result in repression of translation and efficient targeting of the unfolded σ^{32} to degradation. The identification of a high affinity binding site for DnaK within the sequence of region C provides a basis for further experimental investigation of these poorly understood control mechanisms.

Materials and Methods

Electroblot analysis of cellulose-bound peptide libraries

The cellulose-bound peptide scan was prepared by automated spot synthesis (Frank, 1992; Kramer *et al*., 1994) according to the standard machine synthesis protocol (Abimed, Langenfeld, Germany) applying the software DIGEN (Jerini Bio Tools GmbH, Berlin, Germany). For analysis of DnaK binding activity, a cellulose membrane containing a peptide library was incubated at room temperature with DnaK (150 nM) in membrane buffer (170 mM NaCl, 6.4 mM KCl, 0.05% (v/v) Tween, 5.0% (w/v) sucrose, 31 mM Tris-HCl, pH 7.6) for at least one hour. The membrane was washed three times with TBS (170 mM NaCl, 6.4 mM KCl, 31 mM Tris-HCl, pH 7.6) at 4°C and, for transfer of peptide-bound DnaK onto a polyvinylene difluoride (PVDF) membrane, subjected to electroblotting (constant power of 0.8 mA/cm^2) in a semi dry blotter (Phase GmbH, Lübeck) (cathode buffer: 25 mM Tris base, 40 mM 6-aminohexane acid, 0.01% (w/v) SDS, 20% (v/v) methanol; anode buffer I: 30 mM Tris base, 20% methanol; anode buffer II: 300 mM Tris base, 20% methanol). After 20 minutes of blotting eliminating the DnaK background bound to cellulose, the PVDF membrane was exchanged and the transfer was continued. The DnaK transferred to PVDF membranes was detected by DnaK-specific polyclonal rabbit antisera using a chemiluminescence blotting substrate (POD) kit (Boehringer Mannheim).

Peptides and proteins

Peptides were chemically synthesized and purified by HPLC. Peptide concentrations were determined by either quantitative amino acid analysis or calculated after determination of the extinction coefficient (McCarty *et al*., 1995). IAANS-conjugated peptides were made according to manufacturers instructions by mixing peptides with a C-terminal cysteine (~0.15 μmol in water) in 100 mM Hepes-KOH buffer (pH 7.0) containing either 6 M or saturating concentrations of urea with ~0.75 μmol IAANS sodium salt (Molecular Probes, Inc.) from a 1.5 mg/ml stock solution in dimethylformamide. After overnight incubation with agitation at room temperature, free IAANS was separated from peptide by size exclusion chromatography (G25, Pharmacia, 0.7 cm × 30 cm column) and samples (~440μl) were collected in 0.1 M ammonium acetate buffer. Free IAANS was visually observed to be strongly retarded on the column and retained at the top. Concentrations of fluorescent peptide in fractions were estimated by absorbance at 329 nm and pooled fluorescently labeled peptides were determined to be 100% conjugated and free of unconjugated IAANS through HPLC and mass spectrometry analysis. Fluorescently labeled and unlabeled σ^{32}-Q132-Q144-C peptide concentrations were then confirmed by amino acid analysis. DnaK was purified as described (Buchberger *et al*., 1994) and used in Kl/Mg buffer (25 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$). DnaJ was purified according to published procedures (Zylicz *et al*., 1985) with modifications to be presented elsewhere (Schönfeld *et al*., unpublished). Purity of all proteins was >95% as judged by SDS-polyacrylamide gel electrophoresis.

Peptide binding studies

Peptides were screened for the ability to compete with ^{3}H-labeled peptide C of vesicular stomatitis virus glycoprotein (Flynn *et al*., 1989; Buchberger *et al*., 1994; Landry *et al*., 1992) for binding to DnaK and with σ^{32} for binding to DnaJ. In the case of DnaK, the amount of [^{3}H]peptide C remaining bound to DnaK in the presence of competitor peptide was determined after rapid size

exclusion chromatography as described (McCarty *et al*., 1995) under conditions of varying concentrations of competitor over [^{3}H]peptide C. In the case of DnaJ, peptides (300 μM) were added to σ^{32} protein (3 μM) and DnaJ (12 μM) in 130 μl (total volume) of buffer T (20 mM Tris-HCl (pH 7.9), 200 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 5% glycerol) and incubated for 20 minutes at 30°C. DnaJ-σ^{32} complexes were determined after separation from uncomplexed protein through size exclusion chromatography (100 μl applied to a S200 column, Pharmacia, equilibrated in buffer T) and visualisation after immunoblot analysis.

To determine the change in fluorescence upon chaperone binding, IAANS-conjugated peptides (0.5 μM) were mixed with 0.5 μM (final concentrations) of DnaK, DnaJ, ovalbumin (Sigma) or bovine serum albumin (Biomol) in a 500 μl volume of Kl/Mg buffer and incubated for at least 75 minutes at 30°C. The fluorescence emission spectra was determined after excitation at 335 nm, and the emission at the peak of 460 nm was measured in experiments determining the fluorescence response at a single wavelength. The K_D of DnaK for peptide σ^{32}-Q132-Q144-C-IAANS was determined by titration of 0.2 μM peptide with DnaK at concentrations between 0.0125 and 6 μM. Reactions (500 μl) were incubated for at least 120 minutes at 30°C, followed by determination of the fluorescence emission. The fluorescence response is expressed as difference between the measured value and the response in the absence of DnaK. Free DnaK concentration was calculated by subtracting the amount of peptide-bound DnaK from total DnaK, assuming that at saturation levels DnaK was quantitatively bound to peptide. The K_D of binding of unlabeled peptides to DnaK was determined by competition with σ^{32}-Q132-Q144-C-IAANS for binding to DnaK. DnaK (0.1 μM), σ^{32}-Q132-Q144-C-IAANS (0.5 μM) and the unlabeled peptide were mixed in Kl/Mg buffer (500 μl total) and incubated at 30°C for at least 90 minutes. Four or five concentrations of competing peptide were used at concentrations within a tenfold range centering on the inhibition concentration (IC_{50}, estimated by prior measurements over a broad concentration range of competitor peptide) and experiments were repeated at least twice. Within this narrow range of competitor concentrations, the change in reporter peptide fluorescence is linear with competitor concentration and the extrapolated IC_{50} allows calculation of the K_D of competitor peptide as described (Hulme & Birdsall, 1992).

Cloning and sequencing of *rpoH* homologs

Cells of *E. coli* strain KY1608 lacking σ^{32} due to a deletion in *rpoH* were grown at permissive temperature (below 20°C) and infected with a DNA library of each donor (*Enterobacter cloacae, Serratia marcescens, Proteus mirabilis, Agrobacterium tumefaciens, Zymomonas mobilis*), using charomid 9-36 or λ-gt11 as cloning vectors (K.N., H. Yanagi, & T.Y., unpublished). Ampicillin-resistant transformants were selected at 30°C on L agar and those exhibiting high expression of the *lacZ* gene fused to the *groE* gene (λpF13-(*groEp-lacZ*; Kusukawa & Yura, 1988) were analyzed by nucleotide sequencing of the cloned DNAs (K.N., H. Yanagi & T.Y., unpublished). Multiple sequence alignments of σ^{32} and the identified σ^{32} homologs were carried using the ICOT Free Software (Ishikawa *et al*., 1994).

Acknowledgements

We acknowledge H. Bujard for generous support throughout this study, J. Reinstein for help with the K_D determinations, L. Serrano for helpful discussions, and members of the laboratory for critical reading of the manuscript. This work was supported by grants from the DFG priority program ''Molekulare Zellbiologie der Hitzestreßantwort'' and the Fonds der Chemischen Industrie to B.B., and an EMBO fellowship to J.M.

References

Benvenisti, L., Koby, S., Rutman, A., Giladi, H., Yura, T. & Oppenheim, A. B. (1995). Cloning and primary sequence of the *rpoH* gene from *Pseudomonas aeruginosa*. *Gene*, **155**, 73–76.

Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. & Gething, M.-J. H. (1993) Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell*, **75**, 717–728.

Buchberger, A., Valencia, A., McMacken, R., Sander, C. & Bukau, B. (1994). The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171. *EMBO J.* **13**, 1687–1695.

Bukau, B. (1993) Regulation of the *E. coli* heat shock response. *Mol. Microbiol.* **9**, 671–680.

Craig, E. A. & Gross, C. A. (1991). Is Hsp70 the cellular thermometer? *Trends Biochem. Sci.* **16**, 135–140.

Daggett Garvin, L. & Hardies, S. C. (1989). Nucleotide sequence for the *htpR* gene from *Citrobacter freundii*. *Nucl. Acids Res.* **17**, 4889.

Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989). Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science*, **245**, 385–390.

Flynn, G. C., Pohl, J., Flocco, M. T. & Rothman, J. E. (1991). Peptide-binding specificity of the molecular chaperone BiP. *Nature*, **353**, 726–730.

Frank, R. (1992) Spot synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron*, **48**, 9217–9232.

Gamer, J., Bujard, H. & Bukau, B. (1992). Physical interaction between heat shock proteins DnaK, DnaJ, GrpE and the bacterial heat shock transcriptional factor σ^{32}. *Cell*, **69**, 833–842.

Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J. S., Rüdiger, S., Schönfeld, H.-J., Schirra, C., Bujard, H. & Bukau, B. (1996). A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *E. coli* heat shock transcription factor sigma 32. *EMBO J.* **157**, 607–617.

Georgopoulos, C., Liberek, K., Zylicz, M. & Ang, D. (1994). In *The Biology of Heat Shock Proteins and Molecular Chaperones*, (Morimoto, R. I., Tissières, A. & Georgopoulos, C., eds), pp. 209–259, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gragerov, A., Zeng, L., Zhao, X., Burkholder, W. & Gottesman, M. E. (1994). Specificity of DnaK-peptide binding. *J. Mol. Biol.* **235**, 848–854.

Gross, C. A., Straus, D. B. & Erickson, J. W. (1990). In *Stress Proteins in Biology and Medicine*, (Morimoto, R., Tissières, A. & Georgopoulos, C., eds), pp. 167–190, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Grossman, A. D., Straus, D. B., Walter, W. A. & Gross,

C. A. (1987). Sigma 32 synthesis can regulate the synthesis of heat shock proteins in *Escherichia coli*. *Genes Dev.* **1**, 179–184.

Hulme, E. C. & Birdsall, N. J. M. (1992). In *Receptor-Ligand Interactions* (Hulme, E. C., ed.), pp. 63–176, Oxford University Press, Oxford.

Ishikawa, M., Toya, T. & Totoki, Y. (1994). *Proc. Int. Symp. Fifth Generation Computer Systems '94*, pp. 129–138.

Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R., Landgraf, C. & Schneider-Mergener, J. (1994). Combinatorial cellulose-bound peptide libraries: screening tools for the identification of peptides that bind ligands with predefined specificity. *Methods Comp. Methods Enzymol.* **6**, 388–395.

Kusukawa, N. & Yura, T. (1988). Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. *Genes Dev.* **2**, 874–882.

Landry, S. J., Jordan, R., McMacken, R. & Gierasch, L. M. (1992). Different conformations for the same polypeptide bound to chaperones DnaK and GroEL. *Nature*, **355**, 455–457.

Liberek, K. & Georgopoulos, C. (1993). Autoregulation of the *Escherichia coli* heat shock response by the DnaK and DnaJ heat shock proteins. *Proc. Natl Acad. Sci. USA*, **90**, 11019–11023.

Liberek, K., Galitski, T. P., Zylicz, M. & Georgopoulos, C. (1992). The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the sigma 32 transcription factor. *Proc. Natl Acad. Sci. USA*, **89**, 3516–3520.

McCarty, J. S., Buchberger, A., Reinstein, J. & Bukau, B. (1995). The role of ATP in the functional cycle of the DnaK chaperone system. *J. Mol. Biol.* **249**, 126–137.

Naczynski, Z. M., Mueller, C. & Kropinski, A. M. (1995). Cloning the gene for the heat shock response positive regulator (sigma 32 homolog) from *Pseudomonas aeruginosa. Can. J. Microbiol.* **41**, 75–87.

Nagai, H., Yuzawa, H., Kanemori, M. & Yura, T. (1994). A distinct segment of the σ^{32} polypeptide is involved in DnaK-mediated negative control of the heat shock response in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **91**, 10280–10284.

Rost, B. & Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584–599.

Straus, D., Walter, W. & Gross, C. (1990). DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. *Genes Dev.* **4**, 2202–2209.

Straus, D. B., Walter, W. A. & Gross, C. A. (1987). The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. *Nature*, **329**, 348–350.

Straus, D. B., Walter, W. A. & Gross, C. A. (1989). The activity of s^{32} is reduced under conditions of excess heat shock protein production in *Escherichia coli*. *Genes Dev.* **3**, 2003–2010.

Tilly, K., McKittrick, N., Zylicz, M. & Georgopoulos, C. (1983). The DnaK protein modulates the heat-shock response of *Escherichia coli*. *Cell*, **34**, 641–646.

Tomoyasu, T., Gamer, J., Bukau, B., Kanemori, M., Mori, H., Rutman, A. J., Oppenheim, A. B., Yura, T., Yamanaka, K., Niki, H., Hiraga, S. & Ogura, T. (1995). *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor sigma 32. *EMBO J.* **14**, 2551–2560.

Yura, T., Nagai, H. & Mori, H. (1993). Regulation of the heat-shock response in bacteria. *Annu. Rev. Microbiol.* **47**, 321–350.

Zylicz, M., Yamamoto, T., McKittrick, N., Sell, S. & Georgopoulos, C. (1985). Purification and properties of the dnaJ replication protein of *Escherichia coli*. *J. Biol. Chem.* **260**, 7591–7598.

Edited by M. Gottesman

(Received 24 August 1995; accepted 7 December 1995)