A universal protein–protein interaction motif in the eubacterial DNA replication and repair systems

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The interaction between DNA polymerases and sliding clamp proteins confers processivity in DNA synthesis. This interaction is critical for most DNA replication machines from viruses and prokaryotes to higher eukaryotes. The clamp proteins also participate in a variety of dynamic and competing protein–protein interactions. However, clamp-protein binding sequences have not so far been identified in the eubacteria. Here we show from three lines of evidence, bioinformatics, yeast two-hybrid analysis, and inhibition of protein–protein interaction by modified peptides, that variants of a pentapeptide motif (consensus QL[SD]LF) are sufficient to enable interaction of a number of proteins with an archetypal eubacterial sliding clamp (the β subunit of *Escherichia coli* DNA polymerase III holoenzyme). Representatives of this motif are present in most sequenced members of the eubacterial DnaE, PolC, PolB, DinB, and UmuC families of DNA polymerases and the MutS1 mismatch repair protein family. The component tripeptide DLF inhibits the binding of the α (DnaE) subunit of *E. coli* DNA polymerase III to β at μM concentration, identifying key residues. Comparison of the eubacterial, eukaryotic, and archaeal sliding clamp binding motifs suggests that the basic interactions have been conserved across the evolutionary landscape.

The replication of DNA in eubacteria involves many proteins organized into a complex multifunctional machine termed the replisome. A central enzyme is the multisubunit DNA polymerase III holoenzyme. In *Escherichia coli*, and probably in most other eubacteria, the DnaE ortholog (α subunit) is in the core of the replicative polymerase, whereas in many Gram-positive organisms a related enzyme, PolC, is proposed to have this function (1). The processivity of the polymerase is conferred by the direct interaction of the β subunit (clamp protein) of DNA polymerase III (2, 3), with the DnaE (and presumably PolC) subunits. β is loaded onto DNA by a clamp loader comprised of single δ and δ′ subunits and four τ/γ subunits (1). The β dimer thence encircles the DNA without actually binding to it. In addition to DnaE, three other *E. coli* DNA polymerases appear to interact with β. PolB (DNA polymerase II) is involved in DNA repair (4) and the addition of β and the clamp loader increases its processivity *in vitro* (5, 6). Similarly, β and the clamp loader together increase both the processivity (7) and efficiency (8) of DNA synthesis by DNA polymerase IV (DinB). β also appears to play a similar role in the activity of DNA polymerase V (8) (the UmuD′$_2$UmuC complex) and the UmuD subunit has been shown to bind to β (9).

Experimental evidence shows that at least some β-binding proteins can interact productively with β from heterologous species. For example, PolC subunits from *Staphylococcus aureus, Streptococcus pyogenes*, and *Bacillus subtilis* can use *E. coli* β as their processivity subunit (1, 10, 11). In contrast, *E. coli* DnaE cannot use β from the other species (11), the *E. coli* clamp loader complex cannot load *S. aureus* β (11), and the *S. pyogenes* clamp loader complex cannot load *E. coli* β (1).

In the absence of any experimentally identified β-binding sites in proteins, a bioinformatics approach was undertaken to identify putative β-binding motifs. The role of the putative motif was then examined by yeast two-hybrid and peptide-binding experiments with native and modified sequences.

Materials and Methods

Sources of Amino Acid Sequences. Amino acid sequences and alignments were derived from: PSI-BLAST of the protein database at the National Center for Biotechnology Information (NCBI), and BLAST of preliminary sequence data from NCBI at http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html, Institute for Genomic Research at http://www.tigr.org, Department of Energy Joint Genome Institute at http://spider.jgi-psf.org/JGI_microbial/html/, Sanger Center at http://www.sanger.ac.uk/DataSearch/omniblast.shtml, and ERGO at http://wit.integratedgenomics.com/IGwit/. Alignments of available sequences of all members of eubacterial protein families known to bind to β were compiled with manual editing in regions of variable length and sequence. After the identification of the proposed β-binding motif, alignments of the sequences of members of eubacterial families homologous to eukaryotic proteins known to bind to proliferating cell nuclear antigen (PCNA) were compiled from GenBank as described above. Regions containing the proposed β-binding peptide motifs were aligned to maximize matches of a putative β-binding site to the evolving consensus sequence.

Strains. *E. coli* XL-1Blue was the host for all plasmid constructions and source of chromosomal DNA. The pLexA, pB42AD, p8op-lacZ vectors, and yeast EGY48 cells were from the Matchmaker two-hybrid system (CLONTECH).

Yeast Two-Hybrid Assays. The coding region of *E. coli* β (*dnaN*) was amplified by PCR (*Pfu* polymerase) from chromosomal DNA and inserted at the *Eco*RI site of pB42AD to create a translational fusion. To construct various deletions of the *E. coli dnaE*, the appropriate portion of *dnaE* was amplified by PCR and inserted between the *Eco*RI and *Xho*I sites of pLexA for an in-frame fusion. For site- directed mutagenesis, the *dnaE* fragment was cloned into pQE11 (Qiagen, Chatsworth, CA), and mutations were introduced by using the QuikChange system (Stratagene). The PCR-generated fragments containing the mutation then were subcloned into pLexA. To express peptides containing the putative β-binding regions, appropriate regions of *E. coli dnaE*, *polB*, *umuC*, *umuD*, *dinB*, and *mutS* genes were amplified by PCR and fused in-frame to the LexA binding domain through a Gly-Ala-Gly or Ala-Gly-Ala linker. Interactions between β and various LexA fusion proteins were tested in yeast EGY48 containing a *lacZ* reporter gene (EGY48p80p-lacZ). Cotransformants were plated in synthetic complete me-

Abbreviations: PCNA, proliferating cell nuclear antigen; RT, room temperature.

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dium lacking appropriate supplements to maintain plasmid selection and patched onto indicator medium (SG/Gal/Raf/-His/-Leu/-Trp/-Ura with 5-bromo-4-chloro-3-indolyl β-D-galactoside), grown at 30°C, and checked after 36 h for development of blue color. Results were compared with positive (pLexA-53 with pB42AD-T) and negative (pLexA-Lam with pB42AD-T) controls grown in parallel. Cells also were grown to midlog phase in selective media containing glucose or galactose. β-Galactosidase activity is expressed in Miller units. All results are the mean of at least two independent assays with four replicates per assay.

Binding Inhibition Assays. DNA polymerase III subunits α and β were purified by modifications of published procedures using strains containing wild-type *dnaE*$^+$ and *dnaN*$^+$ derivatives of the λ-promoter vector pCE30 (12). The δ subunit was prepared by using strain BL21(DE3)/pLysS/pETδ, essentially as described (13). The α subunit at 20 μg/ml was coated onto 96-well microtiter plates (Falcon flexible plates, Becton Dickinson) in 100 mM Na_2CO_3, pH 9.5 [50 μl/well, overnight at 4°C, or 4 h at 25°C (room temperature, RT)]. Plates were washed in WB3 [20 mM Tris (pH 7.5), 0.1 mM EDTA, containing 0.05% (vol/vol) Tween 20]. This buffer was used in wash steps throughout. Plates were then blocked with Blotto (5% skim milk powder in WB3 100 μl/well, RT) until required, then washed immediately before use.

The peptides were synthesized by fluorenylmethoxycarbonyl chemistry onto preloaded WANG resins that yield free carboxylic acids on cleavage with trifluoroacetic acid (TFA), using standard cycles on a PerSeptive Pioneer Peptide Synthesis System with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as activator. Crude peptides in 6% acetonitrile, 0.1% TFA in water were purified by reverse-phase HPLC (Shimadzu) using a Jupiter C18 semipreparative column (Phenomonex, Belmont, CA), eluted with a gradient to 60% acetonitrile (in 0.1% TFA). Purity and concentration of the peptides were assessed by analytical reverse-phase HPLC, amino acid analysis, and MS.

Peptides in BB14 (20 mM Tris, pH 7.5/10 mM $MgCl_2$/0.1 mM EDTA) were allowed to associate with β (5 μg/ml in BB14; total reaction volume 120 μl) in a 96-well microtiter plate (Sarstedt) that had been pretreated with Blotto (30 min, RT). The β subunit also was incubated in the absence of peptide or in the presence of α at 76.5 μg/ml in BB14. After 1 h (RT), two 50-μl samples were transferred from each well to a corresponding well of the α subunit-coated plates and left for 30 min (RT). Plates were washed and treated with rabbit antiserum to the β subunit that had been diluted 1:1,000 in WB3 containing 10% Blotto (50 μl/well) for 12 min (RT). Plates then were washed and treated for 12 min (RT) with sheep anti-rabbit Ig-horseradish peroxidase conjugate (Silenus, Melbourne, Australia) that had been diluted 1:1,000 in WB3 containing 10% Blotto (50 μl/well). After a final washing step, 1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added (110 μl/well). Color development was assessed at 405 nm by using a plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

The δ-β plate binding assay followed a similar regime, with the following changes: purified δ subunit was coated onto the plate at 5 μg/ml; β at 1 μg/ml was pretreated with the same concentrations of synthetic peptides, and the δ-coated plates were treated with the preformed β-peptide-complexes for just 10 min.

Results and Discussion

Whereas DnaE and PolC are in a family of proteins that does not contain orthologs outside of the eubacteria, PolB proteins are members of a large family of polymerases with representatives in the eukaryotes, viruses, bacteriophages, and archaea (14). The eubacterial PolB family is most similar to the archaeal PolB family, members of which use PCNA (15) [the eukaryotic/archaeal equivalent of β (16)]. The archaeal proteins contain a peptide matching the consensus PCNA-binding motif (17) Qxx-LxxFF (where x is any amino acid) at their carboxyl termini (15). We identified a pentapeptide with the consensus QLsLF (where s is a small amino acid) at, or close to, the carboxyl termini of the known eubacterial PolB proteins (Fig. 1). The similarity of this sequence to the PCNA-binding motif suggested that it may play a role in interaction with β. A similar conserved peptide sequence at the carboxyl termini of members of the eubacterial PolC family also was identified (Fig. 1). The PolC and DnaE families contain homologous core domains, but have different structural organizations and contain some unique domains (18). However, a similar conserved peptide sequence was again identified in members of the DnaE family at the equivalent location to that in PolC, rather than at the carboxyl termini (Fig. 1). The location of the peptide in *E. coli* α is consistent with previous work, which mapped a much larger region of β binding (19). Variants of the peptide sequence also were identified in most eubacterial members of the UmuC and DinB1 families (Fig. 1). However, the level of conservation of the proposed β-binding motifs in the DnaE and DinB1 families was significantly lower than in the PolB and PolC families.

A large number of proteins that bind to PCNA and related proteins has been identified (Table 2, which is published as supporting information on the PNAS web site, www.pnas.org). The eubacteria contain orthologs or close homologs of a number of these proteins, but with the exception of the MutS1 family of mismatch repair proteins (Fig. 1), none contained matches to the proposed β-binding peptide motif. Although interaction of eukaryotic members of the MutS1 family with PCNA has been demonstrated (20, 21), a role for β in eubacterial mismatch repair has not been described.

The frequency of occurrence of each amino acid at each position was determined from putative β-binding peptides present in 250 proteins from six families (Fig. 2). Gln is highly favored (76.4%) at position one, and it is very rarely substituted by Glu (1.6%). At position two, Leu is favored (41.6%), but a wide range of other amino acids are present. At position three, Ser (34%) and Asp (22.8%) are favored, but amino acids with small side chains are also frequently present. In positions four and five, Leu (81.2%) and Phe (76.8%) are also highly favored. Thus, the consensus sequence is QL[SD]LF.

Testing of the peptide motifs for their role in tethering proteins to β was undertaken by yeast two-hybrid and site-directed mutagenesis experiments. Regions of *E. coli* α (DnaE) containing the predicted motif directed expression of β-galactosidase mediated by *E. coli* β. Adjacent regions without the motif did not (Fig. 3). Moreover, mutagenesis of pairs of amino acids in the peptide abolished binding to β (Fig. 3). To examine the generality of the peptide motif peptide sequences and short flanking regions from the *E. coli* DnaE, PolB, UmuC, MutS, and DinB proteins were tested in yeast two-hybrid reactions. Significant expression of β-galactosidase mediated by interaction with β was obtained with all of the peptides, but not with the controls. Even the pLexAPolB(779–783) construct, which contained just the 5-aa motif, gave strong evidence of β binding. The two peptides closest to the consensus gave the highest levels of expression of β-galactosidase. Thus the sequences tested are sufficient to elicit binding of these fusion proteins to β. In contrast, although *E. coli* UmuD has been shown to bind to β *in vitro* (9), the best match to the QL[SD]LF sequence in UmuD (TFPLF) did not mediate significant expression of β-galactosidase, suggesting that the binding of UmuD involves other residues.

To further dissect peptide binding, nonapeptides containing variants of the *E. coli* DnaE motif were synthesized and assayed

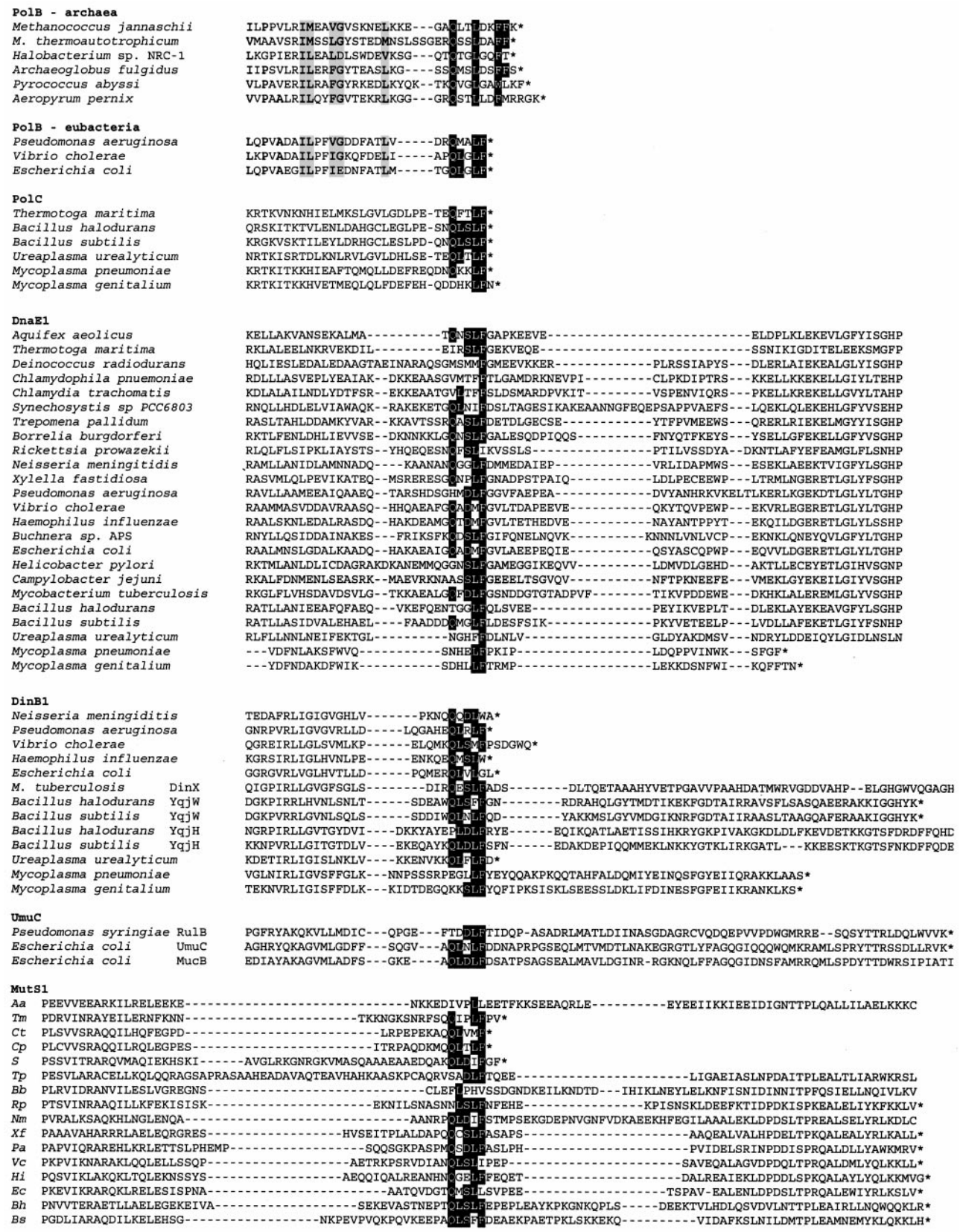

Fig. 1. Alignment of the regions containing the putative β-binding peptides from members of the eubacterial PolB, PolC, DnaE1, DinB1, UmuC, and MutS1 families and the PCNA-binding motif in members of the archaeal PolB family. Representative sequences from fully sequenced genomes, including conserved flanking regions, are shown. Amino acids shown as white with black backgrounds are matches to the β-binding site consensus peptide sequence, amino acids shown with pale gray backgrounds are conservative substitutions in the archaeal and eubacterial PolB sequences. * indicates the terminal amino acid in the protein sequence. For sources of sequences, see Table 3, which is published as supporting information on the PNAS web site.

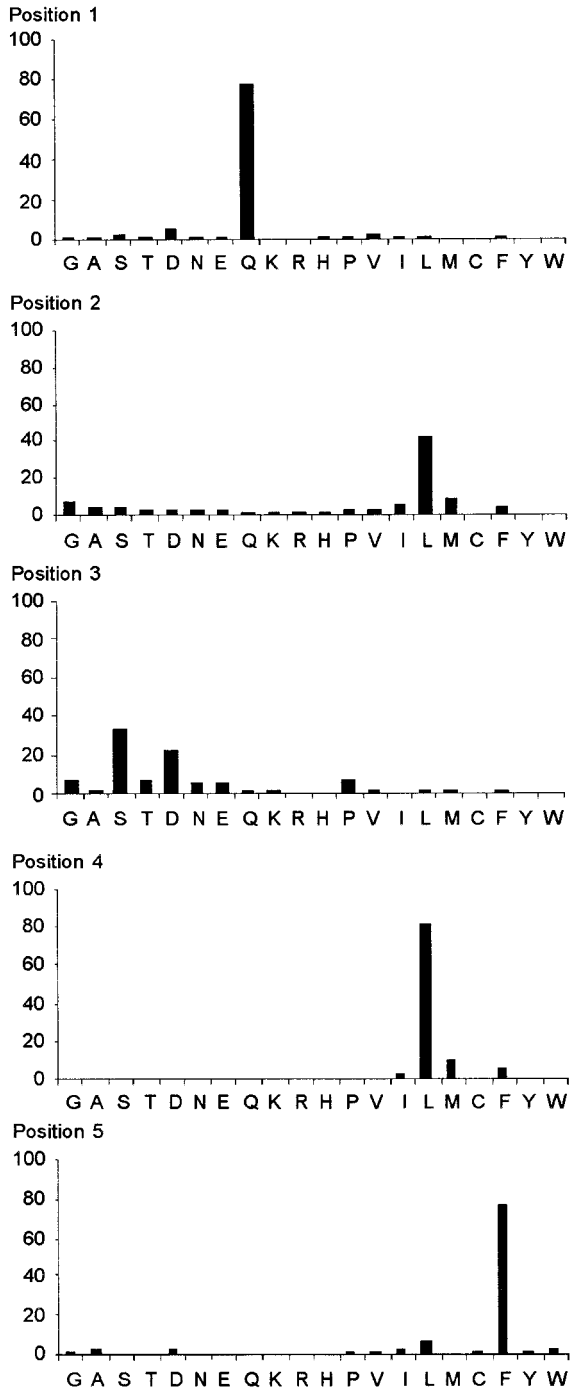

Fig. 2. Distribution of amino acids in putative β-binding peptides. A single peptide sequence with three or more matches to the motif Qxshh (where x is any amino acid, s is any small amino acid, and h is any hydrophobic amino acid) in the appropriate region of the protein from each member of the PolC (22 examples), PolB (15 examples), DnaE1 (72 examples), UmuC (20 examples), DinB1 (62 examples), and MutS1 (59 examples) families of proteins was included in the analysis. Frequency (%) is plotted (as ordinate) for each amino acid at each position of the pentapeptide motif. For a list of sequences used in the analysis see Table 4, which is published as supporting information on the PNAS web site.

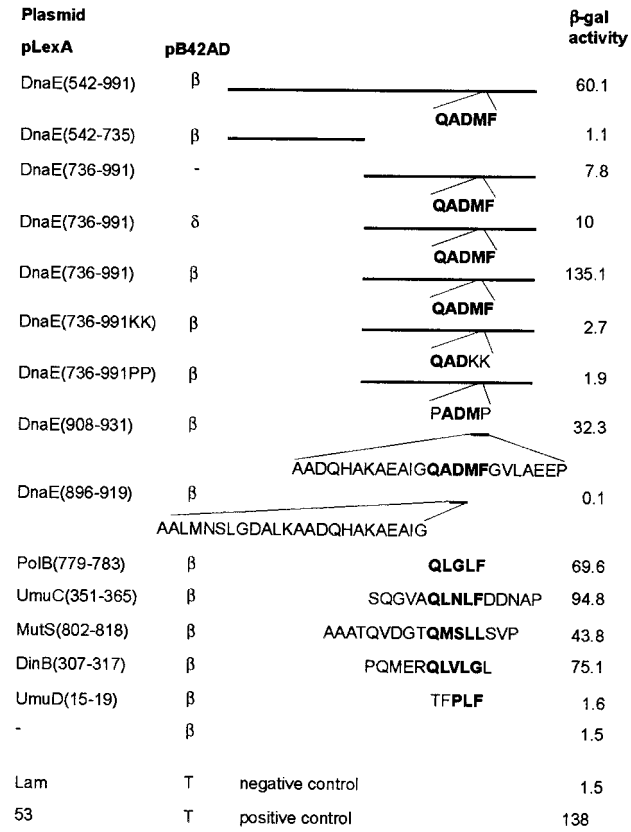

Fig. 3. Yeast two-hybrid analysis of β-binding sites. Numbers in brackets in the plasmid names indicate the amino acid range included in the LexA fusion proteins. Plasmid pLexADnaE(542–991) contains the region of *dnaE* previously identified to contain the β-binding site (19). Plasmid pLexADnaE(542–735) is predicted not to bind to β, based on Kim and McHenry (19). Plasmid pLexA-53 encodes LexA fused to murine p53 protein, pB42AD-T encodes a B42AD domain fused to the simian virus 40 large T antigen, and pLexA-Lam encodes LexA fused to human lamin C.

for their ability to inhibit binding of the α and δ subunits of *E. coli* pol III to *E. coli* β (Table 1). The δ subunit of the clamp loader contacts β during loading (22–24). Ala is present in less than 5% of sequences in any position of the motif (Fig. 2). Alanine scanning of these peptides gave results consistent with bioinformatics analyses. Substitution of the commonly occurring Leu (pep1) for the rarely occurring Ala (pep10) at position two reduced the IC_{50} of α binding $\approx$5-fold (Table 1). Substitution at position four of the very commonly occurring Leu (pep14) for the rarely occurring Met (pep1) reduced the IC_{50} for α binding by a further factor of $\approx$2, whereas substitution of Asp at position three with the more commonly occurring Ser (pep2), increased the IC_{50} for α binding $\approx$35-fold. On the other hand, the equivalent change did not affect the inhibitory capacity of the most strongly inhibitory peptides pep14 and pep13, both of which contain Leu at position two. Consistent with the low frequency of Lys at any position in the naturally occurring sequences (Fig. 2), none of the Lys-containing peptides inhibited binding. Thus, the closer the peptide sequence matches the consensus sequence, the more effectively it inhibits binding of both α and δ to β.

The greater than 75% sequence conservation at positions one, four, and five (Gln, Leu, and Phe, respectively) (Fig. 2) coupled with the experimental data implicates positions two and three in the β-binding peptide, or other regions of the proteins, in modulating binding. The conservation of the amino acids in positions four and five and results of inhibition assays suggested that DLF was the tripeptide most likely to inhibit the binding of α and δ to β. In contrast, the tripeptide QLD, although also containing amino acids highly favored in the pentapeptide, did not contain the highly conserved LF and was predicted not to inhibit binding. These predictions were confirmed (Table 1).

Table 1. Inhibition by peptides of binding of α or δ to β

Peptide	Sequence*	α-binding† IC_{50} (μM)	δ-binding† IC_{50} (μM)
pep10(DnaE)	IG QADMF GV	14.6	218
pep11	IG **A**ADMF GV†	—‡	—‡
pep12	IG QA**A**MF GV	—‡	—‡
pep3	IG QAD**A**F GV	—‡	—‡
pep4	IG QADM**A** GV	—‡	—‡
pep6	IG **P**ADMF GV	—‡	—‡
pep7	IG **K**ADMF GV	—‡	—‡
pep1	IG **Q**LDMF GV	2.8	12.9
pep2	IG QA**S**MF GV	—‡	—‡
pep5	IG QA**V**MF GV	—‡	—‡
pep8	IG QAD**K**F GV	—‡	—‡
pep9	IG QADM**K** GV	—‡	—‡
pep13	IG **Q**L**S**LF GV	1.42	9.5
pep14	IG **Q**L**D**LF GV	1.33	8.8
pep15	**Q**L**D**	—‡	—‡
pep16	**D**LF	135	—‡
pep20	IL LDFGQ VG	—‡	—‡
pep21	DF GVLQL GI	—‡	—‡

*Amino acid substitutions (relative to pep10) are shown in bold.
†Concentration at which binding was inhibited by 50%.
‡50% inhibition of binding was not achieved at 350 μM. Many of the peptides were tested to 1 mM concentration and were found to have an $IC_{50} > 1$ mM in both assays.

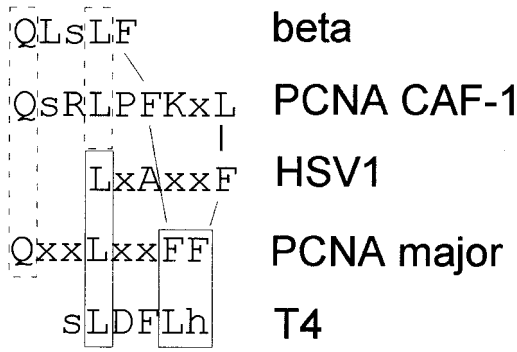

Fig. 4. Comparison of the core sliding clamp-binding motifs. CAF-1 data are from Moggs *et al.* (34) and additional analysis (data not shown), HSV1 data are from Zuccola *et al.* (35), and T4 data are from Berdis *et al.* (33) and Wong and Geiduschek (36). Amino acids for which there is structural data supporting similar roles (35, 37) are in solid boxes; amino acids conserved in position and therefore likely to have similar roles are in dashed boxes; hydrophobic amino acids that are not conserved in position but that contribute significantly to the binding of the peptides are connected by lines.

Thus the pair of hydrophobic residues at positions four and five likely comprises the core of the β-binding motif. Peptides inhibitory to α:β binding were also able to inhibit δ:β binding, although generally IC_{50}s were 5- to 15-fold higher (Table 1). The results with δ and α (Table 1) parallel each other, suggesting that α and δ bind to the same or significantly overlapping sites on β (23). δ proteins do not contain the QL[SD]LF peptide motif, but do contain a related motif (SLF) that is implicated in the binding of δ to β (K.K., B.P.D., and P.A.J., unpublished work).

Specificity of binding was tested by two different means. First, two peptides of identical composition to pep14, but of random sequence, were assayed for inhibition of α:β and δ:β binding. Both peptides exhibited an IC_{50} of greater than 350 μM (Table 1). Second, the inhibitory peptides and random peptides were tested for inhibition of δ:δ' binding. This assay is of similar construction as the α:β and δ:β binding assays. The δ and δ' subunits are known to bind to each other in the clamp loader (25). In this assay all of the peptides exhibited an IC_{50} of greater than 1 mM (G.W., unpublished work).

Our results with the β-binding proteins and peptides demonstrate that the identified motif comprises a significant proportion of the interaction surface on β-binding proteins and is probably functionally equivalent to the PCNA-binding motif (21, 26–31). It is likely that these short motifs are critical for the stability of the total binding interaction and must interact with complementary residues on β. Although the role and importance of additional contacts between β-binding proteins and β is yet to be determined, it appears that they may be less important to core binding than for the PCNA-binding proteins (21, 31, 32). Indeed, whereas a tripeptide can inhibit the *in vitro* binding of α and δ to β, and an octapeptide can inhibit phage T4 polymerase holoenzyme formation (33), at least several flanking amino acids are required in addition to the QxxLxxFF consensus motif for inhibition of binding to eukaryotic PCNA (32). It is noteworthy that eukaryotic PCNA is involved in many more interactions than are the archaeal PCNA or prokaryotic β proteins. The presence of a peptide matching the β-binding motifs is clearly necessary, but not necessarily sufficient, to mediate the binding of a full-length protein to β, because several percent of all proteins in a eubacterial cell would contain pentapeptide sequences that match the observed or permissible variants of the motif. It is likely that both the location in the protein and presentation of the motif is critical for binding to β. Conversely the function of putative (on the basis of their location in the protein) β-binding peptides needs to be investigated to more fully understand the nature of the interaction between the β-binding peptides and β. This is particularly the case in members of the DnaE, DinB1, and MutS families, which exhibit very poor matches to the consensus sequence.

Alignment of the consensus amino acid sequences of the clamp-binding peptides highlights the similarities between the systems (Fig. 4). Several features are apparent; the high level of conservation of Gln at position one among the eubacterial and eukaryotic systems, the conservation of a hydrophobic amino acid (preferably Leu) at the equivalent of position four across all of the motifs, and an additional hydrophobic (frequently aromatic) amino acid at variable locations. The similarities in the binding motifs, and the conservation of a hydrophobic surface of β analogous to the region of PCNA that binds the QxxLxxFF motif in p21 (16, 31) and the region of gp45 that binds gp45-binding proteins (37), suggest that the β-binding proteins bind in a manner that is analogous to the interaction of PCNA-binding proteins and PCNA (31), and of T4 gp45-binding proteins and gp45 (37–39), i.e., primarily via hydrophobic interactions. Consistent with this, mutation of two residues in the carboxyl terminus of β (Pro-363 and Met-364 to Ala) that were predicted by analogy with PCNA to be involved reduced the binding of both α and δ to β (23).

The protein–protein interactions involving the sliding clamp are central to a regulatory network linking DNA replication, recombination, repair, and other cellular processes. At the core of the network are competitive interactions (40). Critical to an understanding of network function is just how such a simple motif, described here, can be central to stabilizing different and competitive interactions. Given its nature it is unlikely that the motif comprises the entire surface for the binding of each interaction partner to the β clamp, rather that the motif is central to the stability of the (substantially hydrophobic) interaction with complementary surface on the clamp protein. The interaction of different partner pairs is likely to be modulated, in terms of affinity and on/off rates, by other interactions involving nearby binding surfaces and, possibly, conformational change in at least one of the partners.

The basic frameworks of both the clamp protein and clamp-binding sites appear across the evolutionary landscape of the eubacteria, archaea, and eukaryotes. The interaction of a DNA polymerase with its sliding clamp is essential for the viability of organisms; the need to preserve competitive interactions of several different proteins with the clamp, and steric constraints imposed by the hydrophobic nature of the key interaction appear to have constrained evolution of the individual components. In the eubacteria the interaction appears to be an eminently suitable target for therapeutic intervention.

Note Added in Proof. Interaction between β and MutS has now been demonstrated; although the site of interaction was not investigated in this work (41).

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