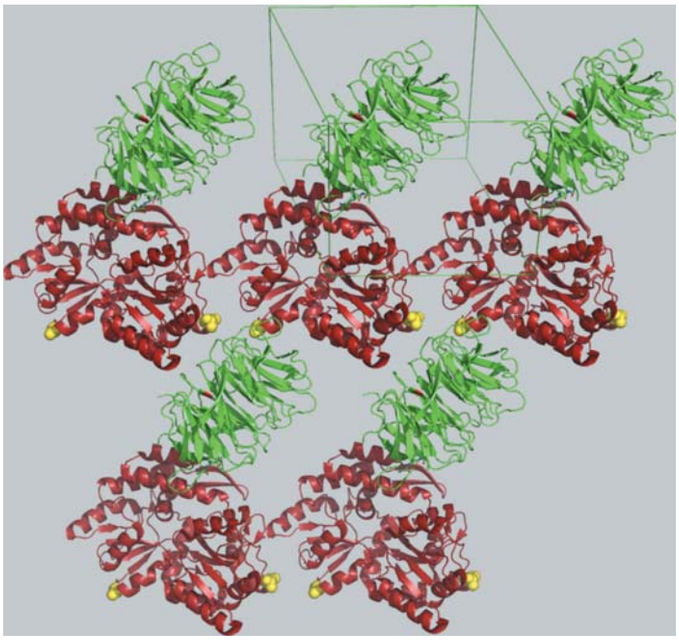

Figure 4.3.5.1
An example of the use of a fused carrier protein in crystallization: the crystal structure of the RACK1 protein (green) crystallized in fusion with an engineered variant of the maltose-binding protein (MBP; red); the major crystal contacts are mediated by MBP (PDB code 3dm0; Ullah *et al.*, 2008). The yellow spheres show alanines introduced by site-directed mutagenesis (see text for further details). Figs. 4.3.5.1 and 4.3.9.1 were generated using *PyMOL* (http://www.pymol.org).

downstream or upstream of the target protein and are often separated from it by a protease-sensitive linker sequence. They are cleaved proteolytically following expression and partial purification of the fusion protein and removed, leaving the isolated target ready for crystallization. However, in some cases the target protein may not be adequately soluble after cleavage or may resist crystallization. One of the possible solutions is to use the intact fusion protein in the crystallization screens in the hope that the carrier protein will both confer solubility on the construct and mediate crystal contacts. Not surprisingly, the canonical carrier proteins, all of which crystallize fairly easily on their own, constitute the obvious first choice. Using this strategy, the DNA-binding domain of DNA replication-related element-binding factor (DREF) was crystallized in fusion with *Escherichia coli* GST (Kuge *et al.*, 1997) and the U2AF homology motif (UHM) domain of splicing factor Puf60 was crystallized as a fusion with thioredoxin (Corsini *et al.*, 2008). A key problem limiting the utility of this technique is the inherent flexibility of a two-domain fusion protein, which is detrimental to its crystallizability. A possible solution to this problem is shortening the linker between the two proteins until a relatively rigid construct is identified (Smyth *et al.*, 2003). This approach was successfully pioneered for maltose-binding protein (MBP), which was used as a fusion chaperone to crystallize the human T-cell leukemia virus type 1 gp21 ectodomain fragment (Center *et al.*, 1998). The same strategy was employed in the crystallization of the ZP-N domain of ZP3 (Monne *et al.*, 2008), the islet amyloid polypeptide (IAPP; Wiltzius *et al.*, 2009) and the MATα1 homeodomain (Ke & Wolberger, 2003). Recently, a genetically modified version of MBP (see below) was used as an N-terminal fusion chaperone to crystallize the signal transduction regulator RACK1 from *Arabidopsis thaliana* (Ullah *et al.*, 2008; Fig. 4.3.5.1). Thus, MBP remains the most successful fusion chaperone for protein crystallization, even though the absolute number of proteins crystallized in this way is still limited.

In addition to the canonical fusion chaperones, which were originally designed as affinity tags, other carrier proteins can be used to assist crystallization. For example, a module made up of two sterile α motif (SAM) domains has been engineered to polymerize in response to a pH drop and was shown to drive the crystallization of 11 target proteins in a pilot study (Nauli *et al.*, 2007). In another example, barnase, a secreted ribonuclease from *Bacillus amyloliquefaciens*, was recently used as a carrier protein for crystallization of the disulfide-rich protein McoEeTI (Niemann *et al.*, 2006).

An alternative to N- or C-terminal fusions is an insertion fusion, in which a carrier protein is inserted into a loop in the sequence of a poorly soluble target. To date, this approach has exclusively been used in membrane-protein crystallization and was initially pioneered for the *E. coli* lactose permease, in which cytochrome b_{562}, flavodoxin and T4 lysozyme were tested as carrier proteins inserted into one of the loops (Privé *et al.*, 1994; Engel *et al.*, 2002). In this specific case none of these variants actually yielded useful crystals and the structure of lactose permease was eventually solved using crystals obtained using a variant containing the C154G mutation which stabilized a single conformation in complex with a lactose analogue (Abramson *et al.*, 2003). In contrast, a similar insertion fusion with T4 lysozyme replacing the third intracellular loop of the β2-adrenergic receptor was highly successful and yielded good-quality crystals that allowed structure determination at 2.4 Å resolution (Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007). This spectacular result attests to the potential of insertion-fusion proteins, but the method is not trivial as the constructs must be carefully evaluated for both structural and functional consequences of the insertion and a number of variants may have to be screened before a suitable one is identified.

4.3.6. Noncovalent crystallization chaperones

Noncovalent crystallization chaperones, *i.e.* engineered binding proteins that produce noncovalent complexes with target macromolecules, constitute an exciting alternative to fusion carrier proteins. Complexes with such chaperones often exhibit enhanced solubility and/or crystallizability in comparison to the isolated targets. The Fab and Fv fragments of antibodies are most commonly used for this purpose (Kovari *et al.*, 1995; Hunte & Michel, 2002; Prongay *et al.*, 1990; Ostermeier *et al.*, 1995; Jiang *et al.*, 2003; Dutzler *et al.*, 2003; Lee *et al.*, 2005). In its canonical version, the technique requires animal immunization with subsequent purification of hybridoma-derived antibodies and their proteolytic digestion to obtain pure homogeneous Fab fragments (Karpusas *et al.*, 2001; Kovari *et al.*, 1995). Alternatively, the Fab fragment can be directly sequenced and a synthetic gene can be used for *E. coli* expression, although this is not trivial owing to the presence of disulfides and two separate polypeptide chains in an Fab molecule. To overcome this bottleneck, a more efficient method of recombinant production of antibody fragments using mammalian HEK 293T has recently been proposed (Nettleship *et al.*, 2008). Another possibility is the use of so-called nanobodies, *i.e.* single-chain fragments derived from camelid antibodies (Koide, Tereshko *et al.*, 2007; Lam *et al.*, 2009; Korotkov *et al.*, 2009). However, this strategy requires immunization of camels or llamas, which is not technically easy.

Regardless of the specific strategy, the use of hybridoma technology and animal immunization is always time-consuming