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Selection of antibody probes to correlate protein sequence domains with their structural distribution

MIKEL VALLE,[1] MANUEL MUÑOZ,[2] LEONOR KREMER,[2] JOSE M. VALPUESTA,[1] CARLOS MARTÍNEZ-A.,[2] JOSE L. CARRASCOSA,[1] AND JUAN P. ALBAR[2]

[1]Department of Macromolecular Structure, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain
[2]Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain

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Abstract

We propose a new approach that permits correlation of specific domains defined by their primary sequence with their location in the structure of complex macromolecular aggregates. It is based on the combination of well-established structural analysis methods that incorporate the use of overlapping peptides on cellulose membranes for the isolation and purification of specific antibodies from a polyclonal antiserum. Monospecific antibodies to the connector protein of bacteriophage ϕ29 were isolated from polyclonal antisera using a new development of the spotscan method. These antibodies can be purified in quantities that allow antigenicity testing in enzyme-linked immunosorbent assays, Western blotting and immunoprecipitations, demonstrating the specificity of this isolation procedure. This approach has allowed us to generate direct antibody probes for immunoelectron microscopy mapping of different connector protein domains in a low resolution three-dimensional epitope map.

Keywords: antibody purification; bacteriophage ϕ29 connector protein; cellulose membrane-bound peptides; epitope mapping; spot synthesis

A key aspect in understanding the structure-function relationship of a macromolecule or macromolecular aggregate is the definition of the three-dimensional (3D) relationship among the distinct structural domains. Computer algorithms generally used to predict the location of domains or epitopes in proteins are relatively successful. Epitope location and size are determined experimentally by screening antisera against large natural or recombinant fragments of specific proteins and thereafter selecting a set of peptides from among the positive fragments. Alternatively, overlapping peptides covering the complete protein length and substitution analogs are screened, allowing identification of specific epitopes, either B or T cell epitopes (Bertoni et al., 1990; Adler et al., 1994; Martens et al., 1995).

Overlapping peptides can be prepared following a number of peptide synthesis strategies, including those that yield peptide arrays bound to solid phases such as polyethylene rods (Geysen et al., 1984) or cellulose membranes (Frank, 1992), or in solution (Houghten, 1985; for a review see Beck-Sickinger & Jung, 1993).

The spot method (Frank, 1992) is a simple, flexible technique for simultaneous parallel chemical synthesis on porous membrane supports such as filter paper, allowing rapid automated distribution of amino acids and low-cost production of large numbers of peptides of use for systematic epitope analysis. The success of this method is reflected in its use in epitope mapping, peptide size analysis, analogue-scan, single Ala substitution analysis (Frank, 1992; Höhne et al., 1993; Commandeur et al., 1994; Reusch et al., 1994; Martens et al., 1995; Stigler et al., 1995; Reineke et al., 1996), analysis of combinatorial libraries for substrate recognition motifs of cAMP- and cGMP-dependent kinases, identification of metal- or nucleic acid-binding peptides (Kramer et al., 1994; Malin et al., 1995; Tegge & Frank, 1995) and the study of the molecular basis of the binding promiscuity displayed by a monoclonal antibody (Kramer et al., 1997). Here we describe a development of the spotscan technology (Frank & Overwin, 1996; Toomik et al., 1996) that uses cellulose membranes with overlapping peptides as immunoadsorbents to isolate and purify specific antibodies from a polyclonal antiserum. Our goal was to employ the resulting epitope-specific antibodies to correlate protein sequence information with epitope location in the protein structure.

As a model system, we have used the *Bacillus subtilis* bacteriophage ϕ29 connector, a dodecameric structure built up of a single protein, p10 (the product of gene 10 of the viral genome) (reviewed by Valpuesta & Carrascosa, 1994; Müller et al., 1997). Viral connectors play an important role in the morphogenesis of complex double-stranded DNA bacteriophages and are essential

Reprint requests to: Juan P. Albar, Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autóonoma de Madrid, Cantoblanco, E-28049 Madrid, Spain; e-mail: jpalbar@cnb.uam.es.

elements in the process of DNA packaging. Underlining their common function, studies carried out in very different viral systems, including T4, T3, T7, ϕ29, lambda, P22, and SPP1, have shown a structurally similar multimeric assembly of a single polypeptide that is modulated by system-specific features. This structural similarity is not reflected in sequence homology, but results from a convergent selection for this structural framework that performs common functions of the connector in the common steps of DNA translocation (Valpuesta & Carrascosa, 1994).

Although the gross 3D structure of the ϕ29 connector assembled from p10 overexpressed in bacteria has been obtained at low resolution (1.8 nm), neither sequence topology nor domain cartography data are available, except for the DNA binding domain obtained by modeling of low-angle X-ray diffraction data (Herranz et al., 1990).

Results

Epitope mapping of bacteriophage ϕ29 connector protein

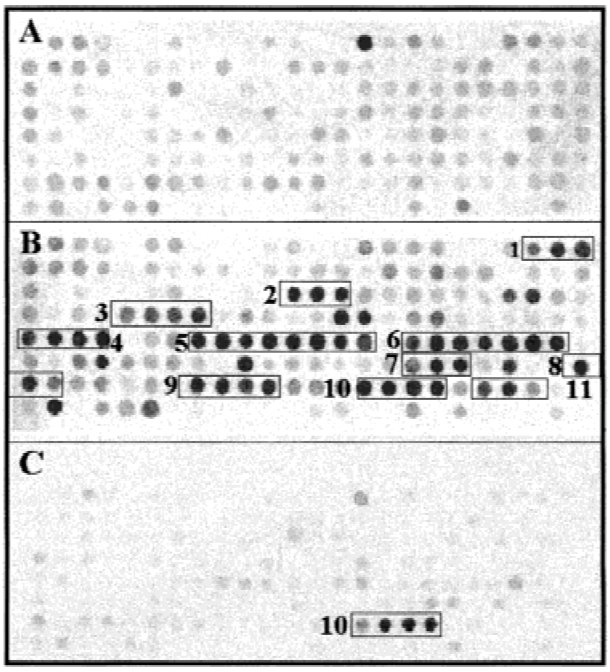
To correlate the sequence data, the position of the functional domains, and the 3D map of the connector, we first defined an epitope map of the ϕ29 connector using a cellulose membrane-based antibody binding assay in which the entire ϕ29 connector protein was represented as a series of overlapping peptides. Polyclonal antiserum was raised in rabbits immunized using the whole native protein.

On a cellulose membrane, we synthesized the entire 308-amino-acid ϕ29 connector p10 protein as 150 overlapping peptides of 10 amino acids each, with a two-amino-acid N- to C-terminus shift. This membrane was used in the antibody binding assays. Pre-immune sera as well as anti-p10 N-terminal peptide antisera were used to control nonspecificity (Fig. 1A) and specificity (not shown) of the cellulose membrane antibody binding assay. With this approach, up to 11 peptide groups were identified, each containing 3 to 5 overlapping peptides (Fig. 1B).

Monospecific antibody purification and characterization

Selected areas of labeled peptides (spots) were excised from the membrane in strips (groups of at least three spots) and tested individually by overnight incubation with whole antiserum. The strips were washed several times with TBS and bound antibodies were eluted as described in Materials and methods. The antibody collected (2–20 μg per round) was assayed using the same antibody binding assay on identical parallel cellulose membranes to monitor the specificity of the adsorption process. For example, the antibody captured from the antiserum by strip 10 reacted with the same peptides on the second membrane (Fig. 1C); no other reactivity was identified in the antibody eluted from this specific adsorption. This example of antibody "fishing" was representative of the adsorption experiments performed, in which eluted antibodies identified only those peptides corresponding to the original membrane group (not shown).

All antibodies recovered in this manner reacted with the native connector protein in an enzyme-linked immunosorbent assay (ELISA) (Fig. 2A). The specificity of this purification process was also confirmed using antibody eluted from the N-terminal strip (1; Fig. 1B), as shown by its ELISA recognition profile of the entire p10 molecule, as well as of a p10 deletion mutant lacking the first



1 ARKRSNTYR INEIQPQKRNRWFIHYLNYL QSLAYQLFEW ENLPPTINPS
51 FLEKSIHQFG YVGFYKDPVISYIACNGALS GQRDVYNQATVFRAASPVYQ
101 KEFKLYNYRD MKEEDMGVVI YNNDMAFPTT PTLELFAAELAELKEIISVN
151 QNAQKTPVLI RANDNNQLSLKQVYNQYEGN APVIFAHEAL DSDSIEVFKT
201 DAPYVVDKLNAQKNAWWNEMMTFLGIKNANLEKKERMVTDEVSSNDEQIE
251 SSGTVFLKSR EEACEKINEL YGLNVKVKFR YDIVEQMRRE LQQIENVSRG
301 TSDGETNE*

Fig. 1. Antibody binding assays on cellulose-bound peptides. Analysis of a series of overlapping decapeptides derived from the ϕ29 p10 connector sequence for binding to a polyclonal anti-p10 antiserum. Pre-immune serum (**A**) and rabbit anti-ϕ29 p10 connector protein antiserum (**B**) were assayed as described in Material and methods, showing the specific recognition pattern by this antiserum. Up to 11 regions of overlapping peptides were sequence-localized. **C:** The recognition pattern obtained when antibodies bound to region 10 in **B** were extracted and assayed with another identical p10 overlapping peptide membrane. The lower panel shows the reactive peptides along the ϕ29 p10 connector sequence, with the common epitope sequence underlined.

four N-terminal amino acids (Fig. 2B). Nevertheless, the antibody did not react with the V8 protease-treated connector, which lacks the first 12 amino acids of the N-terminus. The antibody reacted with a p10 N-terminal synthetic peptide (amino acids 1–14), whereas it did not recognize an unrelated p10 peptide (amino acids 14–26). The highly specific recognition of the connector molecule N-terminal region by this antibody demonstrates the reliability of the method.

Western blot analyses were performed to confirm antibody function and to further substantiate the specificity of the purification process. V8 protease treatment of the ϕ29 connector protein yielded a 33 kDa fragment (p10V8) and small peptide fragments (not shown), whereas trypsin treatment (p10Tryp) yielded a 26 kDa and a 9 kDa fragment (Fig. 2C). The antibody eluted from strip 7 recognized p10, p10V8, and the smaller fragment of p10Tryp, all of which contained the MVTDV sequence, whereas the larger p10Tryp fragment (lacking this sequence) was not recognized (Fig. 2D).

The native protein could also be immunoprecipitated by monospecific antibodies purified by this method. Protein bands corresponding to p10 were precipitated using antibodies eluted from strips 6 and 10 (Fig. 2E). This indicates that these monospecific antibodies have sufficient reactivity with their respective recognition sites as to precipitate the entire native protein.

Spatial configuration of epitopes

In addition to the immunochemical characterization, antibodies eluted from the strips were used to immunolabel the oligomerized native ϕ29 connectors, to correlate linear sequence epitope locations with their spatial arrangement. Images from the immune complexes were studied by electron microscopy. To obtain statistically significant images characterizing each antibody complex, single particle classification techniques were applied to individual projection views, corresponding to side views of the connector labeled with each monospecific antibody. The averages (Fig. 3) revealed the location of the epitopes in the connector structure. The RNA binding domain, which corresponds to an RRM motif type (Donate et al., 1993) (comprising epitope 2), was located near the top of the neck of the connector (Fig. 3B); the region comprising epitope 5 was located in the external subunits that form the widest domain of the connector (Fig. 3C). The C terminus (epitope 10) mapped above that area, in the center of the tronco-conical part of the complex (Fig. 3D). The epitope positions within the 3D structure of the connector are shown in Figure 3E, in which the two-dimensional (2D) projection data have been located on the side view of the 3D connector model.

Discussion

This paper describes a method that combines new approaches for the selection of antibody probes with well-established techniques for antibody characterization and structure determination. This combination allows rapid and reliable correlation of protein sequence data with spatial structure. Overlapping peptides attached to cellulose membranes have been widely used to map linear or discontinuous epitopes. The peptide scanning approach has focused mainly on these types of epitope, since the affinity of separate parts of nonlinear (or discontinuous) (Reineke et al., 1996, 1998; Gao & Esnouf, 1996) protein combining sites to their respective binding partners can be too low for ligand binding detection in standard ELISA techniques. Only the complete and correctly-folded epitope ensures high affinity binding. The polyclonal antiserum induced with the entire p10 dodecameric structure provides a mixture of antibody probes directed mainly toward epitopes exposed on the antigen surface. Although some antibodies to conformational determinants may be overlooked, those reacting specifically with the peptides on the cellulose membrane provide a pattern of reactive regions that must be exposed in the macromolecular structure. Their interaction with the specific antibody probe must be strong, with at least sufficient affinity to be detected in the antibody binding assay.

A basic aspect of this approach is the successful use of overlapping peptides arrayed on cellulose membranes to isolate monospecific antibodies that define the epitope recognition motifs detected by a polyclonal antiserum against the entire native protein. Although other attempts to isolate specific antibodies from polyclonal sera have been described (Tribbick et al., 1991; Beattie et al., 1992), automated amino acid distribution in the preparation of membrane-bound peptides confers an enormous advantage on the spot procedure in terms of reproducibility, accuracy, and speed for the synthesis of large numbers of peptides. The method allows isolation of antibody probes of defined specificity, as shown by the clear information obtained from antibody binding assays using these peptide-bound spot membranes. Specificity is also demonstrated in Western blot using the native and truncated p10 molecules, and in ELISA using these molecules as well as defined synthetic peptides. Although repeated recognition sequences in the dodecameric structure of the antigen may facilitate immunoprecipitation, all antibody fractions isolated from whole serum using this strategy immunoprecipitated the native connector macromolecule, suggesting that these antibody subpopulations must interact strongly with the immunizing protein.

Finally, even in the absence of a detailed atomic model of the connector structure, the images obtained in electron microscopy of the immune complexes generated between these monospecific antibodies and the connector aggregates allowed topographical localization of the interacting protein sequences in a 75 $\times$ 135 Å macromolecular aggregate. The method therefore generates probes for mapping sequence-defined regions in a protein structure that can be used in functional and structural studies, as well as for defining exposed regions of interest of a specific protein. In a broader framework, ligand binding to receptor molecules, defined as overlapping peptides, can easily identify important amino acid sequences for further functional studies.

Materials and methods

Peptide synthesis

Overlapping decapeptides for mapping studies were prepared as described (Reusch et al., 1994) by automated spot synthesis (Abimed, Langenfeld, Germany) onto an amino-derivatized cellulose membrane, immobilized by their C termini via a polyethylene glycol spacer, and N-terminal acetylated. The bacteriophage ϕ29 connector peptides for ELISA experiments, comprising amino acid sequences (1–14) and (14–26), were prepared according to standard Fmoc protocols (Gausepohl et al., 1992) using a multiple peptide synthesizer (Abimed) and analyzed by reverse-phase high-pressure liquid chromatography, amino acid analysis, and mass spectrometry.

Monospecific antibody purification

The areas (spots) on the cellulose membrane identified as reactant regions in the antibody binding assay (see below), once stripped and blocked, were excised and placed in 12 mL polypropylene tubes. Ten milliliters of a polyclonal anti-ϕ29 serum (diluted 1/20 in blocking buffer) were added to the tube and incubated with gentle shaking for 4 h at room temperature or overnight at 4 °C. Membrane strips were then washed three times with Tris-buffered saline/0.5% Tween 20 (TBS-T20) for 30 min and twice with TBS for 15 min, dried on filter paper, placed into Eppendorf tubes and the bound antibodies eluted with 100–500 μL of 0.1 M glycine, pH 3.0 for 5 min. The strips were removed and placed in another 12 mL polypropylene tube containing TBS for reuse. The antibody-containing solution was immediately neutralized with 2 M Tris-HCl, pH 9 (10–50 μL).

Antibody binding to cellulose-bound peptides

The membrane-bound overlapping peptides were blocked overnight with SuperBlock buffer (Pierce, Rockford, Illinois) in TBS-T20. After three washes with TBS-T20, 10 mL of pre-immune or anti-ϕ29 serum (both diluted 1/1,000 in blocking buffer) were added and incubated for 4 h at room temperature. After three washes with TBS-T20, membranes were incubated with an alka-

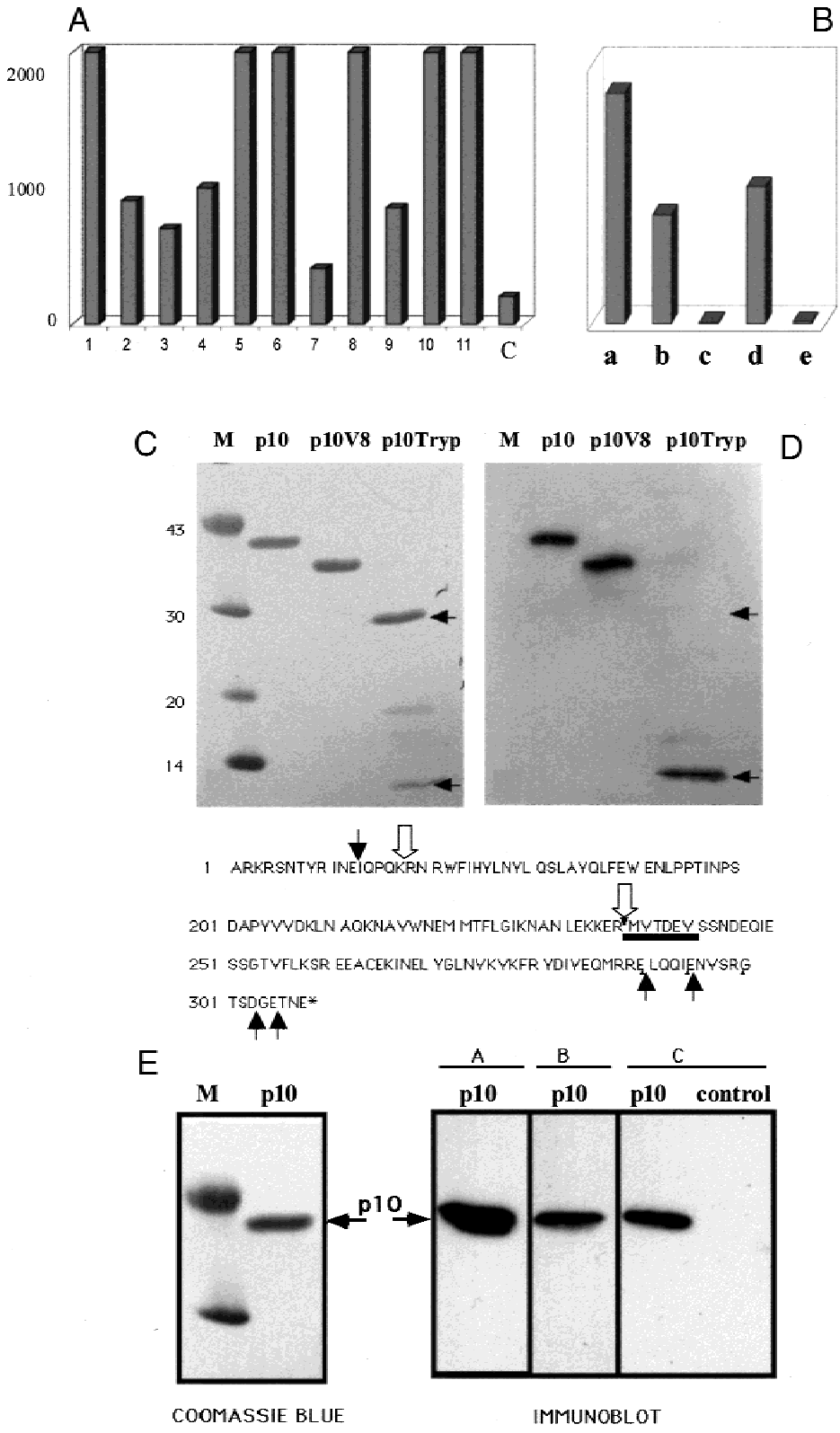


Fig. 2. *See caption on facing page.*

line phosphatase-labeled anti-rabbit antibody (diluted 1/5,000; Gibco-BRL, Gaithersburg, Maryland) for 2 h at room temperature, after which an AP color development system (BioRad, Hercules, California) was applied using nitro blue triazolium and bromochloroindolyl phosphate. Under these conditions, color development times could be selected to avoid nonspecific background. Membranes were reused by stripping all adsorbed material in consecutive washing steps with water, dimethylformamide, 8 M urea, 0.1% SDS, and 0.1% β-mercaptoethanol, as described (Frank & Overwin, 1996).

Direct ELISA studies

Connector protein was obtained following the procedure described (Ibáñez et al., 1984). All assays were performed in 96-well microtiter plates (Costar, Roskilde, Denmark) coated with 2.5 μg/mL proteins: recombinant or wild-type ϕ29 connector p10, V8 protease-treated recombinant p10 (p10V8), connectors assembled from p10ΔN4 (lacking the first four N-terminal amino acids), or ϕ29 p10(1–14) and ϕ29 p10(14–26) peptides in phosphate-buffered saline (PBS) containing 0.3 M NaCl (to avoid connector aggregation). Plates were incubated overnight at 4 °C (or 1 h at 37 °C), then blocked with 1% bovine serum albumin (BSA) for 1 h at 37 °C; wells were washed three times with PBS between assay steps. Antibodies (diluted in PBS containing 0.5% BSA) were added and incubated for 1 h at 37 °C, followed by peroxidase (PO)-labeled goat anti-rabbit Ig (Amersham, Little Chalfont, United Kingdom) for 1 h at 37 °C. The reaction was developed 5 min at room temperature with o-phenylenediamine dihydrochloride and the reaction terminated with 50 μL of 2 M sulfuric acid. The A_{492} was determined in a Titertek Multiskan spectrometer (Labsystems, Helsinki, Finland).

Western blotting

Connector proteins were protease treated (*Staphylococcus aureus* V8 endoproteinase Glu-C or trypsin) at a 20:1 (w:w) ratio for 2 h at 37 °C. Reactions were terminated using phenylmethylsulfonyl fluoride (PMSF) for trypsin or dichloroisocoumarin for V8-treated samples. Proteins were separated in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the fragments transferred to a polyvinyldene fluoride (PVDF) membrane (Immobilon, Millipore, Mississippi) in 10 mM 3-(cyclohexyl amino)-1-propane sulfonic acid buffer containing 10% methanol and 0.1% SDS. Membranes were blocked by incubation with 1% BSA in PBS for 1 h at room temperature, washed, then incubated for 2 h with isolated antibodies diluted 1/100 in PBS containing 1% BSA and 0.05% Tween-20. After three washes with T-PBS, membranes were incubated with a PO-labeled anti-rabbit antibody (Amersham) for 2 h at room temperature, developed using a chemiluminescence system (Amersham) and exposed to standard X-ray film.

Immunoprecipitation assays

The ability of strip-extracted monospecific antibody preparations to precipitate native p10 connector protein was studied. Aliquots of recombinant ϕ29 connector p10 (0.2 μg in 200 μL of 0.4 M NaCl-PBS containing 1% BSA) were preincubated with anti-rabbit Ig-agarose beads (15 μL) for 2 h at room temperature with gentle shaking. After centrifugation (1,500 $\times$ g), protein-containing supernatants were incubated with antibody preparations (diluted 1/10) in the same buffer overnight at 4 °C, followed by anti-rabbit Ig-agarose (15 μL/tube, 1 h, room temperature). After four washes with 0.4 M NaCl-PBS, immunoprecipitates were separated in 12% SDS-PAGE and transferred to PVDF membranes as above. Western blot analyses were developed with a polyclonal mouse anti-ϕ29 p10 connector antibody and a PO-labeled goat anti-mouse Ig as second antibody.

Electron microscopy analysis of immune complexes

Averaged images of connector-antibody complexes were obtained using various antibody preparations extracted from membrane-bound peptide membrane strips. Monospecific antibody extracted from the membrane (for example, 5) was incubated with connectors (10–20 μg/mL) in 0.3 M NaCl for 2 h at 37 °C. Samples were negatively stained with 2% uranyl acetate and visualized in a JEOL 1200 EX II, using low electron dosage. Images were obtained

Fig. 2. (*facing page*) Biochemical characterization of the monospecific antibodies. **A, B:** ELISA recognition of connectors by antibodies purified from peptide membrane. Plates were incubated at 4 °C overnight with a solution of connectors or with synthetic peptides at 2.5 μg/mL in PBS containing 0.3 M NaCl (to avoid connector aggregation). **A**: Recognition of p10 connectors by the monospecific antibodies corresponding to the sequences shown in the lower part of Figure 1. Bar C represents a nonspecific background control. The *Y*-axis shows milli absorbance units at 492 nm. **B:** Recognition pattern of the monospecific antibody against the N-terminal peptide 1 using (1) wild-type p10 connectors, (2) connectors assembled from p10ΔN4 (lacking the first four N-terminal amino acids), (3) proteinase V8-treated connectors (lacking the first 13 N-terminal amino acids), (4) synthetic peptide of the first 14 N-terminal amino acids of p10, and (5) synthetic peptide of protein p10 amino acids 14–26. **C, D:** Immunoblot using monospecific polyclonal antibodies eluted from peptide membranes. The connectors were protease-treated at a 20:1 (w:w) ratio for 2 h at 37 °C. Reactions were terminated using PMSF for trypsin or DCI for samples treated with *S. aureus* V8 endoproteinase Glu-C. The reaction products were separated in 12% SDS-PAGE. **C**: The Coomassie blue-stained gel. Relative mobility markers are shown in lane M. Lane p10 is control p10 connector protein. p10V8 shows the main fragment generated by V8 protease treatment of p10 connectors. p10Tryp shows the two main fragments generated by trypsin treatment of p10 connectors. The protease cleavage sites are indicated in the p10 sequence (below) using filled (V8) and open arrows (trypsin). **D**: The identification of the cleavage peptides by immunoblot using the monospecific polyclonal antibody selected by peptide sequence 7 (underlined). **E**: Immunoprecipitation of native connectors by monospecific antibody. Samples of native connectors were incubated with antibody for 2 h at room temperature, precipitated using anti-rabbit Ig, then separated in PAGE. Left panel shows Coomassie blue-stained peptides. Arrow marks the position of p10. Lane M are marker proteins (ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa). Right panel shows Western blot of immunoprecipitated samples developed using a mouse anti-ϕ29 p10, as described in Materials and methods. (A) Connectors incubated with polyclonal antibody raised against a synthetic p10 N-terminal peptide. (B) Connectors incubated with monospecific antibody 6. (C) Connectors incubated with monospecific antibody 10. Lane labeled "control" was loaded with the precipitate from connectors incubated with normal rabbit serum.

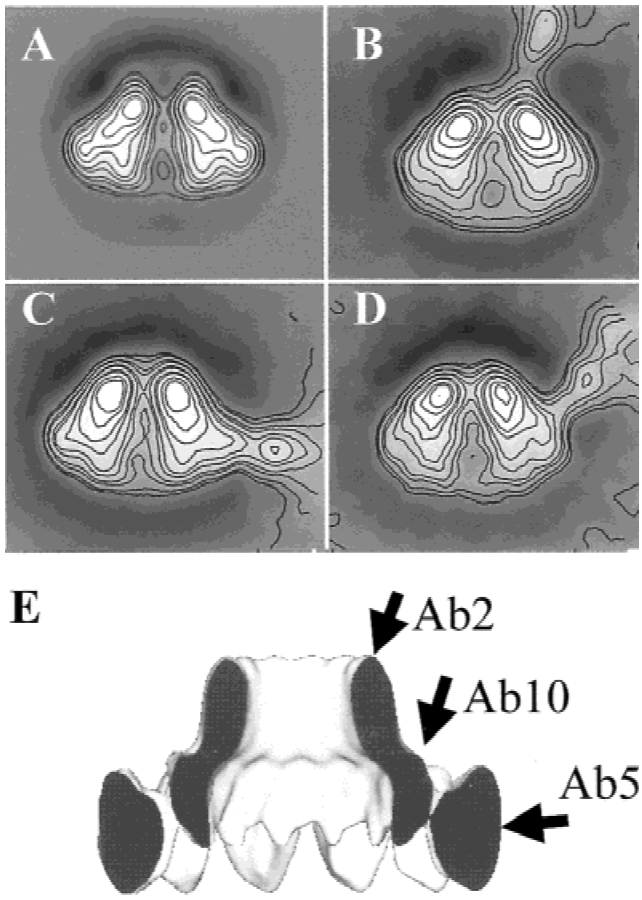


Fig. 3. Averaged electron microscope images of connector-antibody complexes. Samples containing immune complexes were negatively stained and images processed as described in Materials and methods. **A**: Contour gray level curves superimposed on the image of the averaged side view of a control connector obtained from 400 particles at 3 nm resolution. **B**: Averaged side view of complexes between the connector and antibody 2 (RNA binding motif region), obtained from 105 particles at 3 nm resolution. The epitope recognized by this antibody is labeled by the averaged Fab, shown as a clear, stain-excluding region. **C:** Averaged side view of the complex with antibody 5 (206 images). **D:** Average side view of the complex with antibody 10 (carboxy terminal) (107 images). **E**: Location of the different epitopes in the 3D map of the connector based on the two-dimensional projection averages of the immune complexes and their comparison with the side view of the 3D connector map. The diameter of the widest domain of the connector side view represents 13.5 nm.

using a slow scan CCD and processed using the XMIPP program package (Marabini et al., 1996). Individual images were aligned using cross-correlation and pattern-free alignment methods (Kohonen, 1990; Donate et al., 1992). Homogeneous populations for averaging were selected using self-organizing maps (Penczek et al., 1992; Marco et al., 1996). The 3D model of the reconstructed ϕ29 connector was obtained by cryoelectron microscopy at a resolution of 1.2 nm using tilting series of samples containing 0.9% sucrose and 0.1% uranyl acetate (J.M. Valpuesta, J.J. Fernández, J.M. Carazo, & J.L. Carrascosa, unpubl. obs.).

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