Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes

G. Smolenski, P. A. Sullivan,† S. M. Cutfield and J. F. Cutfield

Author for correspondence: J. F. Cutfield. Tel: +64 3 479 7836. Fax: +64 3 479 7866. e-mail: johnfc@sanger.otago.ac.nz

Biochemistry Department, University of Otago, PO Box 56, Dunedin, New Zealand

The recently discovered secreted aspartic proteinase multi-gene (SAP) family in *Candida albicans* has complicated assessment of proteolytic activity as a factor in the onset and development of *Candida* infections. Differential expression of the SAP genes under various conditions, as well as possible variation in the properties of the individual isoenzymes, have consequences for immunological detection, for targeted drug design and possibly for pathogenicity. It is therefore important to be able to monitor Sap isoenzyme profiles in different strains of *C. albicans* cultures, and to know the biochemical properties of each isoenzyme. We have employed a simple purification protocol based on strong anion exchange chromatography for the direct analysis of *C. albicans* Sap isoenzymes from culture filtrates, as well as recovery of individual Sapl, Sap2 and Sap3 products. In the case of Sapl, this involved development of an overexpression system using the pEMBLyex4 vector transformed into *Saccharomyces cerevisiae*. The *C. albicans* strains ATCC 10231 and 10261 were shown to produce different ratios of Sap2 and Sap3 under the same conditions. Analysis of all three purified proteins by gel electrophoresis, immunoblotting and proteinase assays which were designed to evaluate pH dependence, thermal stability and substrate specificity revealed similar but distinct properties for each isoenzyme. Although Sap3 was shown to be antigenically more similar to Sap2 than was Sapl, it was less similar in terms of thermal stability and activity at low pH, being more stable and more active.

**Keywords:** aspartic proteinases, *Candida albicans*, virulence factors, recombinant protein, isoenzymes

INTRODUCTION

An ongoing interest in the secreted aspartic proteinase (Sap) activity of *Candida* spp., and *Candida albicans* in particular, and its possible role as a virulence factor, has been heightened by the discovery of a multi-gene SAP family (references include Staib, 1969; Cutler, 1991; Ray et al., 1991; Rüchel et al., 1992; Magee et al., 1993; Monod et al., 1994; White & Agabian, 1995). Amongst the eight SAP genes identified so far, the protein products of four of these (Saps 1, 2, 3 and a putative Sap8) have been characterized to various extents. Indeed, the isoenzyme now known as Sap2, which represents the major product secreted *in vitro* by the yeast form of many strains of *C. albicans*, was first isolated and identified as an acidic proteinase by Remold et al. (1968). It was subsequently characterized further by Rüchel (1981) and since then a number of reports on the purification, activity and substrate specificity of this enzyme have appeared (Negi et al., 1984; Kaminishi et al., 1986; Ray & Payne, 1990; Bannerjee et al., 1991; Yamamoto et al., 1992; White et al., 1993; Morrison et al., 1993a, b; Fusek et al., 1994; Tsushima et al., 1994). The SAP2 gene itself was cloned and sequenced by Wright et al. (1992), being the second gene in the family to be identified. Most recently, the crystal structure of Sap2 from two different strains of *C. albicans* was determined (Cutfield et al., 1995; Abad-Zapatero et al., 1996), revealing details of the binding site for peptide-based
inhibitors. These structural studies showed that the binding site contained some amino acids that varied amongst the Sap family members, which might affect individual specificities.

Identification of the SAP1 gene (Hube et al., 1991) preceded characterization of its protein product by White et al. (1993), who also identified the SAP3 gene and its protein. They further showed that the switching strain WO-1 secreted the Sap2 isoenzyme from white cells whereas the opaque cells expressed Saps 1, 2 and 3 (White & Agabian, 1995). Hube et al. (1994) observed the expression (transcription) of Saps 4, 5 and 6 in several strains only during serum-induced yeast-to-hyphal transition. It is now clear that the pattern of Sap isoenzyme production in C. albicans depends on strain, cell type and environmental factors. More speculative at this stage is the suggestion that the differential transcription of the various SAP genes is associated with adherence and colonization of host tissue.

Originally motivated by the need to produce milligram quantities of individual Sap isoenzymes for crystallization studies, we have now established a heterologous expression system for these proteins, as well as modifying existing purification protocols to produce a simple procedure for monitoring Saps 1, 2 and 3 in culture filtrates and for recovering pure protein. This has enabled us to compare some biochemical properties of these three isoenzymes from one strain and, where applicable, to compare them with other published results.

METHODS

Strains and plasmids. Candida albicans strains used were ATCC 10231 and 10261. Saccharomyces cerevisiae AH22 (Leu2· His2) (Hinnen et al., 1978) was the host for recombinant Sap, while Escherichia coli DH5α was the host for cloning the SAP1 gene. The plasmid pSapl (referred to as CaPrA in Magee et al., 1993) was provided by B. Hube (Universität Hamburg, Germany) and contains 5·4 kb C. albicans ATCC 10231 DNA including the SAP1 open reading frame. The plasmids used for cloning and expression were pBluescript II KS(+/-) to give pGS1. The plasmid pGS1 was digested with BamHI and Ksp6321 to give two distinguishable fragments, each of approximately 1800 bp. These were isolated and digested with BglII to produce 1800 bp of genomic DNA and two fragments (1100 and 700 bp) of vector DNA. The 1800 bp product was gel-purified, end-filled using DNA polymerase I (Klenow fragment) and T4 DNA polymerase, and ligated into the Smal site of pBluescript II KS(+/-) (pGS2). The DNA containing the SAP1 gene was excised from pG52 as a 1400 bp fragment using BamHI and XbaI and directionally subcloned into the yeast expression vector pEMBLyex4 (pGS3). This fragment contained 14 bp of 5’ sequence, the 1173 bp SAP open reading frame and about 210 bp of 3’ sequence. The correct orientation of the subcloned fragment was confirmed by restriction mapping and sequencing. Strain AH22 was then transformed with pGS3 (Burgess & Percival, 1987) with selection for Leu· on minimal medium plus histidine (100 mg l-1).

Purification of secreted aspartic proteinases

(a) Sap2 and Sap3. C. albicans ATCC 10231 (or 10261) was grown as a starter culture overnight in 200 ml YPD medium in a 1·1 flask at 30 °C, then added to 101 YBD medium and incubated for 18 h at 30 °C with an aeration rate of 10 l min-1. After cell harvesting, the medium was adjusted from pH 3·2 to pH 6·5 with 6·M NaOH and concentrated to 500 ml by ultrafiltration (10000 Da molecular mass cut-off). Following a buffer exchange with 10 mM sodium citrate (pH 6·8), the concentrate was applied to a pre-equilibrated DEAE-Sephadex column (24 cm x 2·5 cm) and eluted at a flow rate of 60 ml h-1 over a 1·1 linear gradient from 10 to 300 mM sodium citrate (pH 6·3). Fractions containing the enzyme activity were pooled, concentrated to 10 ml by ultrafiltration, buffer-exchanged with 20 mM bis-Tris/HCl (pH 6·0) and filtered through a 0·45 μm membrane to remove any particulate matter. Aliquots (1 ml) of this enzyme concentrate were applied to a pre-equilibrated Pharmacia Mono Q HR 5/5 column and eluted with a linear gradient from 20 mM bis-Tris/HCl (pH 6·0) to the same buffer plus 300 mM KCl at a flow rate of 10 ml min-1 under the control of a Waters Millipore 650 Advanced Protein Purification System. Individual peak fractions containing Sap activity were concentrated, buffer-exchanged, and then subjected to another Mono Q chromatography step in order to achieve maximum purity. Purified enzyme samples were routinely stored frozen at -20 °C in 100 mM Tris/HCl (pH 6·7). Protein concentration was determined by the modified Lowry procedure of Peterson (1977), using BSA as a standard.

(b) Recombinant Sap1. Recombinant Sap1 was induced in 1 l cultures by the addition of 24% (w/v) galactose using the conditions described previously for heterologous expression in S. cerevisiae (Chambers et al., 1993). The medium was harvested 12–24 h post-induction, concentrated 20–50-fold and buffer-exchanged into 20 mM bis-Tris/HCl (pH 6·0) prior to Mono Q ion exchange chromatography as described above for Saps 2 and 3.

Assays for enzyme activity

(a) BSA. Based on the assay of MacDonald & Odds (1980), enzyme activity was measured spectrophotometrically (Pharmacia-LKB Ultraspec II spectrophotometer) following the digestion of BSA as substrate. A typical reaction mix containing 500 μl 20% (w/v) BSA in 50 mM sodium citrate (pH 3·2), 100 μl 30 mM sodium citrate (pH 3·2) and 200 μl of culture supernatant was incubated for 30 min at 37 °C. The reaction was stopped by adding 200 μl 2 M perchloric acid and incubated on ice for 15 min. Precipitated protein was removed.
M6000A pumps, a 680/440 systems controller and detector with 100 ng Sap, total volume 100 µl, for a specified time. The N-terminal sequencing was performed with a gas-phase reaction was stopped by addition of 100 µl 0.1 M NaOH. Tyrosine-containing peptides in the neutralized sample were determined with Folin’s reagent according to the Lowry method. To each 150 µl sample, 750 µl reagent consisting of 20% (w/v) sodium carbonate in 1 M NaOH, 10% (w/v) copper sulphate and 20% (w/v) sodium tetraborate was added and incubated at room temperature for 10 min before addition of 75 µl 50% diluted Folin’s reagent. After 30 min incubation at room temperature, the change in absorbance was determined. One enzyme unit for this system corresponds to the production of 1 µg tyrosine min⁻¹ measured as an A₅₂₅₀ of 0.058.

(c) pH and thermal stability activity profiles. To examine pH/ activity profiles using either substrate, a range of buffers was employed, each at 50 mM concentration. These were glycine, citrate, acetate and Tris/HCl. For measurements of thermal stability, samples (0-1 ml) of enzyme were heated for 15 min at a specified temperature and then assayed at 37 °C as above.

PAGE and immunoblotting. Proteinase samples containing 25 mM Tris/HCl (pH 7.0), 10% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol and 0.05% bromphenol blue were heated at 100 °C for 5 min prior to SDS-PAGE. Electrophoresis (Mini-Protein II; Bio-Rad) was for 2-3 h at 150 V (constant voltage) using the discontinuous buffer system described by Schagger & van Jagow (1987). Alternatively, native PAGE was performed without the addition of any of the denaturing components in the above system. Following electrophoresis, samples were transferred to nitrocellulose, incubated with a 1:3000 dilution of rabbit anti-proteinase antiserum (Ross et al., 1990) and visualized using the detection system of Blake et al. (1984). The antigen was a highly purified preparation of Sap2 from strain ATCC 10261.

N-terminal sequencing. Enzyme and peptide samples (~100 pmol) were applied to Immobilon PVDF membrane or Polybrene-coated glass-fibre disks, respectively. Automated N-terminal sequencing was performed with a gas-phase instrument (Applied Biosystems model 470A-R/120A/610A). Fragments of Sap proteins for internal sequencing were obtained by treatment with V8 protease from Streptobacillus aureus at an enzyme to substrate ratio of 1:30 (w/w), in 100 mM ammonium bicarbonate for 50 min at 37 °C.

Reverse-phase HPLC. For analysis of substrate specificity, separations were carried out using a Waters Chromatography binary gradient system consisting of a U6K injector, two M6000A pumps, a 680/440 systems controller and detector with extended wavelength module. A wide-pore C8 silica-based Bakerbond column (4.6 mm x 250 mm) was employed in conjunction with a linear gradient, from 0-1% trifluoroacetic acid (TFA) to 80% acetonitrile/002% TFA. Insulin B-chain (15 µg) in 50 mM citrate buffer (pH 3.2) was digested with 100 ng Sap, total volume 100 µl, for a specified time. The reaction was stopped by addition of 100 µl 10% TFA/20% acetonitrile, and was applied immediately onto the column.

RESULTS

Purification of Sap2 and Sap3

An outline of the purification scheme used for obtaining homogeneous preparations of Saps 2 and 3 from the culture medium of C. albicans strain ATCC 10231, or indeed from other strains, is shown in Fig. 1. It is based on the well-known ability of aspartic proteinases, with their generally low isoelectric points, to bind to anion-exchange resins, and was first used by Remold et al. (1968) for Candida Sap. Two such steps have been employed in this study: the ‘traditional’ DEAE chromatography (in this case as per Rüchel, 1981 and Wright et al., 1992) followed by a much faster and higher resolution separation with Mono Q (strong anion exchanger). However, it was not necessary to include the DEAE step as can be seen in Fig. 2, where culture filtrates, after concentration and buffer exchange, were applied directly to the Mono Q column. In the interests of column preservation and for higher protein loadings, the preliminary DEAE step is, however, recommended for removal of some extraneous protein. Sap activity was located in two sharp peaks identified by N-terminal sequencing as Sap2 (the larger) and Sap3, respectively. Further confirmation was obtained by sequencing internal fragments generated by treatment with V8 protease. A second round of Mono Q chromatography was considered important for the high degree of purity required for crystallization trials and for comparative studies of individual isoenzymes.

The one-step Mono Q procedure allowed rapid analysis.

Peptide products were analysed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Finnigan MAT) and N-terminal sequencing.

Fig. 1. Flow diagram of the purification scheme for either recombinant Sap or native Sap isoenzymes. The DEAE-Sepharose step is recommended for recovery of milligram quantities of protein but may be bypassed if rapid analysis of Sap isoenzyme profiles is required.
G. SMOLENSKI and OTHERS

0.10
0.08
0.06
w
q
0.04
0.02
0.0
50
1000
200
600
400
200
0
KCl (mM)

Fig. 2. Mono Q ion exchange chromatography of concentrated culture filtrate from C. albicans strains 10261 (a) and 10231 (b). Sap activity was eluted when a linear gradient of 0-300 mM KCl/20 mM bis-Tris/HCl (pH 6.0) was applied; flow rate was 1 ml min⁻¹.

Expression of Sap1 in S. cerevisiae

Heterologous expression of recombinant Sap1 was based on a fragment of DNA containing the full-length open reading frame identified originally from a genomic library of ATCC 10231 DNA (Hube et al., 1991). The fragment was inserted into the multicopy yeast expression vector pEMBLyex4, allowing expression in S. cerevisiae strain AH22. The purification of Sap1, the main protein species secreted into the medium, was simple, involving just the one chromatography step with Mono Q (Fig. 1), to give a twofold purification of enzyme activity from the filtrate and a 69% total recovery. The purified enzyme had a specific activity of 3.2 U mg⁻¹ using BSA as a substrate, which is less than for Saps 2 and 3 (5 U mg⁻¹) prepared from C. albicans as above. Although the final yield of protein was low [1.1 mg (1 culture)⁻¹], the ease of purification largely compensated for this. Proof of the correct identity of the secreted protein was secured by N-terminal sequencing, mobility on SDS-PAGE, Western blotting and pH dependence of proteinase activity. Yeast cells transformed with vector only showed no Sap activity and no immunoreactivity. The partial sequence obtained (QAIPVTLNNE...) was indicative that S. cerevisiae had correctly processed the precursor form of the enzyme. Using the same heterologous expression system (A. D. Scadden & P. A. Sullivan, unpublished), recombinant Sap2 isoenzyme has also been produced and this was found to possess the same specific activity as the native protein. Therefore it can be reasonably expected that the recombinant Sap1 so produced will have similar activity to native Sap1.

Table 1. Purification of Saps 2 and 3 from C. albicans ATCC 10231

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Culture medium</td>
<td>9780</td>
<td>1213</td>
<td>3917</td>
<td>0.3</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Concentrate</td>
<td>506</td>
<td>873</td>
<td>874</td>
<td>10</td>
<td>73</td>
<td>3.2</td>
</tr>
<tr>
<td>DEAE-Sephael</td>
<td>170</td>
<td>424</td>
<td>178</td>
<td>3.9</td>
<td>35</td>
<td>12.6</td>
</tr>
<tr>
<td>Concentrate</td>
<td>10·1</td>
<td>351</td>
<td>79</td>
<td>4.4</td>
<td>29</td>
<td>14.2</td>
</tr>
<tr>
<td>Mono Q Sap2</td>
<td>13·1</td>
<td>197.7</td>
<td>39·5</td>
<td>5·0</td>
<td>163</td>
<td>16.2</td>
</tr>
<tr>
<td>Sap3</td>
<td>6·2</td>
<td>93·2</td>
<td>18·6</td>
<td>5·0</td>
<td>7·7</td>
<td>16·2</td>
</tr>
</tbody>
</table>

and 18 mg Sap3 being obtained from a 10 l culture. These data also show that, using the BSA assay, the specific activities of Saps 2 and 3 were very similar (5 U mg⁻¹) and that a 16-fold purification of the culture filtrate activity had been achieved. By comparison, 41 mg Sap2 and only 1·8 mg Sap3 was obtained from strain 10261 grown under the same conditions. The specific activity for Sap2 was the same as in 10231; however, for Sap3 it was 20% lower (4 U mg⁻¹). Similar relative activities were seen when haemoglobin was used as substrate.
Analysis of three *Candida* Sap isoenzymes

Comparative properties of Sap1, Sap2 and Sap3

The three Sap isoenzymes from strain 10231, purified as above, are readily distinguishable by anion exchange chromatography (Fig. 3) and also by native gel electrophoresis (Fig. 4a). Both of these methods exploit the overall charge on the molecule and it can be seen that the order of increasing negative charge was Sap3 > Sap2 > Sap1. Fig. 3 shows that if all three Saps were secreted at the same time, they could be easily identified by their elution times and quantified relative to each other from the $A_{280}$ profile, remembering that the Sap3 peak should be reduced by a factor of 1.3 with respect to both Sap1 and Sap2. Analysis by SDS-PAGE (Fig. 4b) shows a running order Sap1 > Sap2 > Sap3, corresponding to apparent molecular masses of 38, 41 and 42 kDa, respectively, which are 3–6 kDa higher than the molecular masses deduced from their sequences. It is interesting to note that if the gel recipe was altered to increase the bis:acrylamide ratio, then Saps 2 and 3 ran parallel. There was no evidence (from carbohydrate analysis, amino acid sequencing and crystallography of Sap2) for any significant glycosylation which would explain the slower than expected migration that nevertheless is consistent with other gel analyses of Saps.

Western analysis of the three isoenzymes (Fig. 4c) using a polyclonal antibody preparation directed against purified Sap2 (Ross *et al.*, 1990) clearly showed that there were common epitopes amongst them, the order of reactivity being Sap2 > Sap3 > Sap1. Quantification of the Western blots by soft laser densitometry indicated that Sap3 was 90% and Sap1 65% immunoreactive in comparison to Sap2.

The pH dependence of the isoenzymes was determined using a range of buffers between pH 2.0 and 7.0. Similar profiles were observed in all three cases (Fig. 5a), although Sap3 showed significantly higher activity below pH 2.5 with both BSA and haemoglobin substrates. Above pH 7, all three Saps were essentially inactive. The pH optima using either substrate were all in the range 3.2–3.5.

The upper temperature for thermal stability using either substrate was observed to be 45 °C for each of the three isoenzymes (Fig. 5b). Both Saps 1 and 2 lost approxi-
...isoenzymes.

G.

no major differences in specificity amongst the three

With this particular substrate, therefore, there are

have demonstrated that the enzyme can cut at many sites

natural and synthetic substrates. These experiments

indicated that the A_5 profiles are quite complex, with

most of the peptide peaks being common to all three

enzymes but varying in height (Fig. 6). Analysis of some

of these peaks showed that a favoured cleavage site lies

between B24 Phe and B25 Phe (peak a is the B25–30

peptide), but also indicated several other sites close

together in the region B12–B15 (Val-Glu-Ala-Leu),
similar to the original finding by Remold et al. (1968).

With this particular substrate, therefore, there are

no major differences in specificity amongst the three

isoenzymes.

DISCUSSION

This paper presents (a) the first report of recombinant

Sap production, (b) the use of a straightforward protocol

for recognizing and purifying Sap isoenzymes and (c) a

comparison of some fundamental properties of Saps 1, 2

and 3 from a non-switching strain, ATCC 10231.

The SAP1 gene from C. albicans strain 10231 was

expressed as a correctly processed (secreted) recombi-
nant protein in S. cerevisiae, albeit at a modest level,

although no special effort was made to optimize the

process. As measured, the specific activity of the pure

Sap1 was lower than that of Saps 2 and 3 obtained from

cultures of C. albicans 10231. Purification of Saps 2 and

3 was almost as simple as for Sap1, the key step being the

rapid, high-resolution ion exchange step with Mono Q.

Analysis of fractions following Mono Q chromato-

graphy showed that under proteinase-inducing condi-
tions (YBD medium), the Sap activity secreted by 10231

(or 10261) was associated with Saps 2 and 3 only. Sap1

expression has so far only been observed in the opaque

phase of the switching strain WO-1 and it is not yet clear

under what conditions, if any, 10231 could express the

SAPI gene product. Using oligonucleotide probes

specific for Saps 1, 2 and 3, Northern analyses of both

10231 and 10261, grown under the same conditions as

for Sap protein production, were consistent with the

protein results obtained in this study (R. J. Wright

& P. A. Sullivan, unpublished). An earlier report by Wright

et al. (1992) had indicated some expression of Sap1 in

10261 but the full-length probe used in that experiment

would have allowed cross-hybridization to occur. It is

clear then that evidence from both mRNA and secreted

protein levels shows that Sap2 is the major isoenzyme

expressed and that strain 10231 is a significantly greater

producer of Sap3 than is 10261 under the conditions

used.

Reasonably pure preparations of Sap2 (known previ-

ously under a variety of names, including CAP, PRA

and PEP gene products) have long been attainable. Most

of these protocols have incorporated a DEAE chromato-

graphy step following the original work of Remold et al.

(1968), together with at least one other step. This group

combined gel filtration with DEAE ion exchange as did

Rüchel (1981), who subsequently introduced pepstatin-

linked affinity chromatography (Rüchel et al., 1982). A

pseudo-affinity step followed by gel filtration was

employed by Ray & Payne (1990), while most other

preparations have incorporated variations on the above

themes (summarized by Morrison et al., 1993b). Analy-

sis of purity of these Sap preparations has been carried

out by various methods, including SDS-PAGE, immuno-

blotting and isoelectric focusing with confirmation by

N-terminal sequencing in some cases. Given that Saps 1,

2 and 3 are of similar molecular mass (approximately

36 kDa), they migrate close together in denaturing gels

and are not easy to discriminate in mini-gels, especially

so Saps 2 and 3 (see also White et al., 1993). We have

![Fig. 5. (a) pH profiles and (b) thermal stability profiles for Saps 1 (■), 2 (▲) and 3 (○) from strain 10231. Activity assays used BSA as substrate. Similar results were obtained with haemoglobin as substrate. Points shown are the mean of triplicates.](image_url)
shown that non-denaturing gels offer better discrimination and high-resolution fast anion exchange chromatography even more so.

It was recently pointed out that despite the many and varied studies on the biochemical properties of Sap, there were inconsistencies in the results, in part due to lack of identification of the various isoenzymes (White & Agabian, 1995). Bannerjee et al. (1991) characterized two activities in cultures of SC5314 which presumably corresponded to Saps 2 and 3, and some earlier studies had also suggested more than one activity, including those of Remold et al. (1968). The first clear demonstration of the presence of three distinct isoenzymes was by White et al. (1993), who showed that a single strain, WO-1, is able to secrete Saps 1, 2 and 3 and that the levels of these isoenzymes were dependent on environmental factors. We have also isolated the three isoenzymes (Sap1 as a recombinant), from a non-switching strain in this case, and compared several of their basic functional properties. In terms of pH profile, there was little difference, though Sap3 appears to be considerably more active at pH 2. Of greater interest perhaps is the higher thermal stability exhibited by Sap3, but more stringent denaturation tests would need to be carried out in order to confirm that this isoenzyme possesses greater structural stability. As far as the specificity of substrate cleavage is concerned, the results found with insulin B-chain reiterate earlier findings of broad specificity, regardless of isoenzyme type. Some preliminary results using synthetic peptides indicate that there may be some better defined specificity preferences among the Saps but even so this would seem to be of little importance in vivo.

Although phylogenetic analysis of Sap proteins by Monod et al. (1994) has shown that Sap1 and Sap3 display similar sequence similarity to Sap2, the immunoblotting results indicated that the anti-Sap2 antibodies recognized Sap3 better than they did Sap1. The possibility that the Sap2 preparation used as antigen (Ross et al., 1990) may have contained a small amount of Sap3 is unlikely as it had been extensively purified to give a clean amino acid sequence and a single band on SDS-PAGE. Examination of the known three-dimensional structure of Sap2 (Cutfield et al., 1995) offers a possible explanation for the different responses. There are several exposed regions on the surface of the molecule that are possibly antigenic and that vary in sequence among the isoenzymes, with Sap1 showing more radical substitutions than Sap3, relative to Sap2. For example, the loop region 49–53 in Sap1 has two basic amino acids not seen in the others; the loop region 241–244 also has a different charge distribution and there are a number of exposed single-site substitutions involving change in charge or in polarity.

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