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Ready display of antigenic peptides in a protein 'mimogen'†‡

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Given the dependence of much modern biology upon the use of antibodies as tools and reagents, their variability and the often associated lack-of-detail about function and identity creates experimental errors. Here we describe the proof-of-principle for a potentially general, versatile method for the display of antigens in a soluble yet standard format on a lateral protein scaffold that mimics normal epitopes in a protein antigen (a 'mimogen') and confirm their utility in phosphorylation-dependent recognition by specific antibodies.

The general adoption over >35 years of gel-based analytical techniques for the identification of proteins using specific antibodies (Abs)[1,2] (so-called "Western blotting") has led to its use as a mainstay technique in biological sciences. There is now a vast array of Abs available from different sources, with variable amounts of associated characterization data.[3,4] For example, commercial sources may have little or no data on breadth of target epitopes/antigens ('cross-reactivity') or antibody sequence from which serious scientific issues may arise. It also reduces productivity as end users frequently need to screen many antibodies before a reliable one can be found for a particular biomarker and/or system. The low standards of Ab characterization also contribute to the significant economic cost of irreproducible pre-clinical research, which has been estimated at $28bn per annum for pre-clinical research in the USA alone.[5,6] Most researchers use western blotting as a qualitative technique capable of identifying a biomarker of interest, or as a semi-quantitative technique to examine the relative quantity of the same biomarker in separate biological specimens. However, with the inclusion of an appropriate calibration standard, western blotting can potentially generate quantitative data for a given biomarker (enabling estimation of, for example, protein amount and even extent of structural alteration).[7–9] It also validates the utility of the Ab against that calibrant in a direct manner. A general method that would allow fast and efficient screening of antibody binding specificity and calibration through the display of target epitopes on a standard protein platform would therefore be a potentially powerful tool.

We considered that one method for generating epitopes displayed in a scaffold with protein-like properties (allowing ready use of *e.g.*, gel electrophoresis, protein quantification dyes as well as other associated well-developed protein techniques) would be to create a generalizable protein platform onto which epitope peptides could be laterally grafted covalently (Fig. 1). This would avoid the necessity to create a bespoke protein appropriately displaying the epitope on each associated occasion that a calibrant was required (through, *e.g.*, creation of a fusion

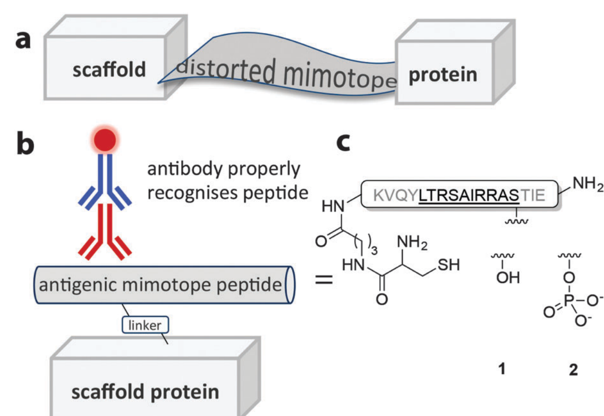

Fig. 1 General concept (b) for the precise 'lateral' display of antigenic mimotope peptides **1** and **2** on a protein scaffold that would avoid potential issues of context dependency created by current linear, fusion display methods (a), such as perturbation of secondary structure.

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† JC founded and has a financial interest in Badrilla Ltd. JC is an inventor on a patent related to mimogens. MS, JC, BGD developed the concepts. RV, MS, JC, BGD, designed the experimental systems. MS, RV, ZJB performed experiments. Mimogen western analysis was performed at Badrilla and by ZJB. All analysed the results. RV, MS, JC, BGD wrote the manuscript. All read and commented on the manuscript.

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protein or other display-dependent methods)[10] and would permit the display of epitopes with precise post-translational modifications as required. It would also avoid any associated complications that might stem from the influence of the display method or scaffold on secondary/tertiary structure that might arise from linear display in this way (Fig. 1a). We envisaged that such a 'lateral display' system might allow the efficient mimicry of epitope(s) as part of a greater protein as a pseudo-antigen that might be called a 'mimogen' (a mimicking antigen, by reference to 'mimotopes'[11]§). Advantageously, a common protein platform would allow similar, modular methods for construction and common functional (*e.g.*, macromolecular) properties (Fig. 1b); this would allow rapid and economic construction of large numbers of such mimogens, as is required for large-scale systems biology research programmes.

As a test system we chose one of the most vital and functionally wide-ranging post-translational modifications: protein phosphorylation. Phospholamban (PLN) regulates the sarco(endo)plasmic reticulum calcium ATP-ase (SERCA) pump in cardiac and skeletal muscle cells through a mechanism governed by its phosphorylation state.[12–14] As such, PLN plays a crucial role in the physiological regulation of cardiac contractility and hence a vital role in human biology. PLN is a 52 amino acid membrane protein that exists in monomeric and homopentameric forms that can be difficult to generate as readily controllable standards or calibrants.[15] The simple structure and known sites of phosphorylation suggested PLN as an ideal candidate for the mimogen approach.

Phosphorylation site-specific Abs have been produced to PLN, which discriminate between Ser-16 phosphorylated and unphosphorylated PLN.[16] These and other Abs (that recognise PLN in all states of phosphorylation) have been used to gain a qualitative and semi-quantitative understanding of the role PLN plays in stress responses in the heart.[17] The epitope sequences of Abs to PLN overlap considerably: a monoclonal Ab A1, that is used routinely to measure total protein, recognises residues 7–16 on PLN, where Leu7 is the most important determinant.[12] Putative phospho-specific antibody PS-16 was raised to an 11 amino acid fragment of PLN (residues 9–19 RSAIRRAS$_{16}$TIE) peptide built of residues 9–19, where Ser at position 16 was phosphorylated.[16]

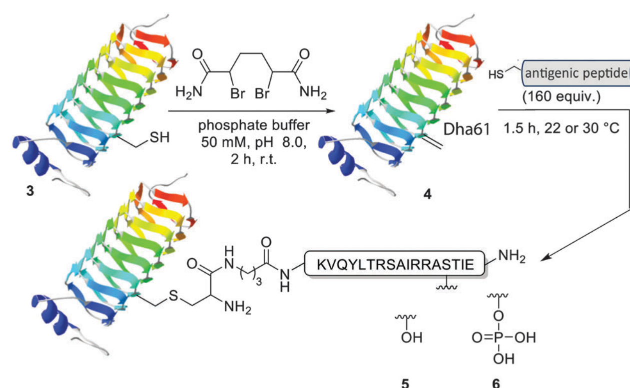
Peptides **1** and **2** (see Fig. 1 for sequences) containing a key segment of PLN in unmodified and phosphorylated states, respectively, were designed and synthesized using essentially standard solid-phase peptide synthesis techniques (see below and SI). As an appropriate scaffold for the attachment of these antigenic mimotopes a protein with regular motif, moderate molecular weight and good stability as well as non-perturbing activity and structure was required. The fused pentapeptide repeat protein (PRP) Np275/Np276[18] (here referred to as Np276 or Npβ) was chosen as a potentially ideal scaffold that met these criteria; in particular, its ordered beta-sheets arrayed in a repeating cuboid geometry[19] we reasoned could be exploited for controlled display of antigens, with the potential for extended, multivalency and even distance-control. In addition, it displays minimal non-specific binding properties, likely as a function of its unusual structure, balanced hydrophobicity/philicity and lack of singular motifs.

1 and **2** were precisely displayed on Npβ through a "tag-and-modify"[20] approach; here through post-expression installation of a selectively reactive chemical group into the protein, the "tag", followed by the selective modification of this new group. Our group and others have developed versatile protocols for the selective formation of the dehydroalanine (Dha) tag in proteins genetically, biosynthetically and chemically[21–24] as a usefully selective conjugate electrophile.

Model scaffold mutant protein Npβ-G2F-M61C (**3**) was expressed in *E. coli* BL21(DE3) (at 16 °C) as a designed mutant resistant to N-terminal *N*-gluconoylation[25] (by virtue of G2F) and readily converted to Dha61 following expression (by virtue of M61C as a sole Cys). After expression, **3** was treated with 2,5-dibromohexanediamide (DBHDA)[24] to yield the 'tag'-bearing scaffold **4** protein Npβ-G2F-Dha61 (Scheme 1 and Fig. 2). Peptides **1** and **2** proved to be challengingly bulky conjugate nucleophiles, larger than many previous nucleophiles reacted with Dha in proteins. A thiol group with a relatively low pK_a of ~8 (1–2 units lower than the typical pK_a of alkyl thiols[26]) was designed into **1** and **2** *via* a cysteinylated-spacer-motif (based upon gamma-aminobutyric acid) to favour conjugate addition as a thiolate or readily deprotonated thiol. Advantageously, the entire unit was prepared from commercially-available building blocks using standard Fmoc-based methods.

Precise conjugation between the antigenic mimotope **1** or **2** and protein scaffold **4** (Scheme 1) was tested under conditions of varying temperature, buffer systems (phosphate, tris/HCl and borax) and pH (values between 8.0 and 10.0) (Table 1). At lower pH, phosphate buffer proved superior to tris/HCl or borax buffer (Table 1) but conversion was more positively affected by a higher pH. Notably, addition of denaturing reagents (*e.g.*, 3 M guanidine·HCl) did not lead to an improvement in product formation, suggesting that reactivity was not dependent on the protein scaffold tertiary structure and that the scaffold is non-interfering in both chemistry and display. Optimal conditions (borax buffer (50 mM), 30 °C, pH 10) allowed ready conversion of Npβ-G2F-Dha61 (**4**) to desired unphosphorylated protein-peptide conjugate Npβ-PLN$_{2-19}$ **5** (>90%, Table 1, Entry 8 and Fig. 2).

Application of the same procedure also allowed successful conjugation of the phosphorylated antigenic mimotope peptide **2** onto **4** to give protein–peptide conjugate **6** under the same reaction conditions (Scheme 1) without side-product formation, albeit with more sluggish conversion requiring prolonged reaction



Scheme 1 Conjugation of antigenic mimotope peptide to Npβ scaffold.

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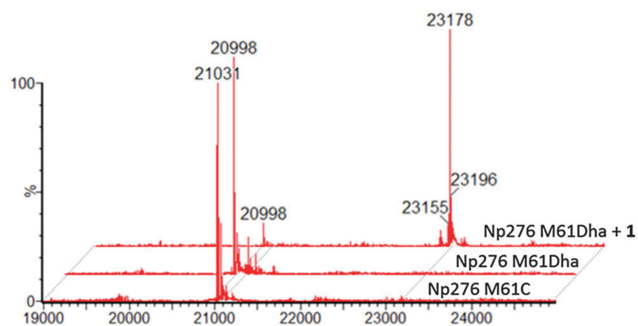

Fig. 2 LCMS analysis of conversion of Npβ-G2F-M61C (**3**) to Npβ-Dha61 (**4**) and then reaction to give antigenic mimogen peptide-protein conjugate **5**.

Table 1 Optimization of conjugation of Npβ scaffold with antigenic peptides

Entry	Peptide	Buffer (50 mM)	pH	T (°C)	Y^a (%)
1	**1**	Phosphate	8	30	56%
2	**1**	Phosphate (+guanidine 3 M)	8	30	35%
3	**1**	Tris/HCl	8	30	21%
4	**1**	Tris/HCl	9	22	34%
5	**1**	Tris/HCl	9	30	63%
6	**1**	Borax	9	22	39%
7	**1**	Borax	10	22	60%
8	**1**	Borax	10	30	93%
9	**2**	Phosphate	8	30	9%
10	**2**	Borax[b]	10	30	43%

[a] Determined by LCMS, reaction time 1.5 h. [b] Reaction time 5 h.

times (Table 1 and ESI‡). Advantageously, due to the large mass difference between **4** and **6** and the lack of side products, **6** could be readily separated from **4** by HPLC or size exclusion chromatography, allowing possible recycling.

With unphosphorylated and phosphorylated antigenic mimogen conjugates **5** and **6** in hand, we tested both 'mimogens' in crude western blot analysis to examine the binding specificity of antibodies A1 (total PLN) and PS-16 (anti-PLN pSer16). The monoclonal Ab A1 to PLN recognised both unphosphorylated (PLN) and phosphorylated PLN (pPLN) proteins (sarcoplasmic reticulum (SR) samples: control and PKA treated respectively; Fig. 3a, lanes 2,3) and phosphosphorylated and unphosphorylated mimogens **5** and **6**. However, it displayed a clear preference for unphosphorylated mimogen **5** (Fig. 3a, lane 4 *vs.* 5) consistent with the intended efficient mimicry by **5** of unphosphorylated PLN. The binding specificity of the pSer16 phospho-specific Ab PS-16 was examined on the same western blot membrane, after removing the A1 complexes. This Ab discriminated between unphosphorylated and Ser16 phosphorylated (pSer16) protein (Fig. 3b, lanes 2,3) and mimogens **5** and **6** (Fig. 3b, lanes 4,5), providing a signal only with phosphorylated epitope samples, again consistent with the designed mimogen system. The naked mimogen scaffold (Npβ-G2F-Dha61) was not detected by either antibody, further confirming its suitability as a display scaffold. Thus, even in this crude, re-used format, immediate and clear readout could be obtained directly from quick and ready reactive use of the scaffold to create mimogens.

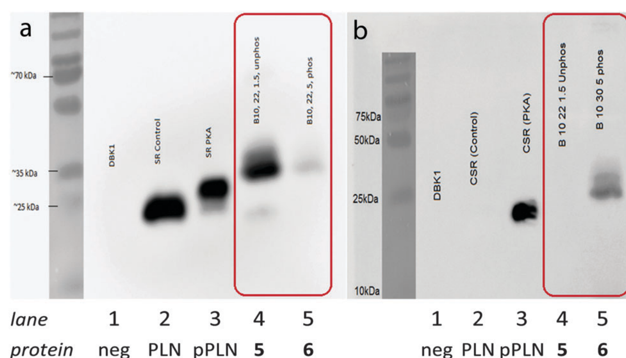

Fig. 3 Western blot analysis showed binding of (a) Ab A1 and (b) Ab PS-16 towards five proteins: negative control protein DBK1 (lane 1), PLN (lane 2), phosphoPLN (lane 3), and crude mimogen conjugates **5** (lane 4) and **6** (lane 5). Reference mass markers have been superimposed. Gels shown directly from image scan with only coded labels added as registration markers. Red boxes highlight mimogens.

Together, these initial data confirmed the design and utility of this protein scaffold system as a potentially suitable 'neutral' display system for antigenic detection of model mimotope peptides that here allow clear detection and discrimination of the modification state of a single contextual phosphorylation site (Ser16 in PLN), despite not being displayed in a native protein context.

One advantage of the Dha tag is its versatility in diverse elaboration, making it a potentially useful strategy for the elaboration of a single protein scaffold in several functionally discriminatory directions (here pSer *vs.* Ser). Here, a readily produced peptide motif could be directly conjugated following automated synthesis with a stable but reactive protein scaffold **4** to give differentiated peptide-protein conjugate mimogens directly. Although high levels of conjugation (>90%) were achievable, we also demonstrated that since the chemistry for conjugation is usefully clean, gel electrophoresis provides a method for separation that allows the use of even partially reacted samples. As such, it is a proof-of-principle that may offer a method for 'quick-and-easy' access to protein-like antigens (termed here 'mimogens'), even for incompletely reacted samples, without requiring specialist knowledge of protein chemistry methods. In this way, we believe that this method might not only provide the 'non-expert' with valuable antigenic alternatives for proteins not currently accessible by direct expression methods but also a 'kit-like' system for quick and cheap reference alternatives in western blotting experiments[7–9] or other protein antigen contexts.

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Notes and references

§ Whilst the term antigen has gained more generalized usage with broader context, we use here the term antigen in a manner consistent with its original definition (it originally described a structural molecule that binds specifically to an antibody) and that adopted by IUPAC (Gold Book; a substance that stimulates the immune system to produce a set of specific antibodies and that combines with the antibody through a specific binding site or epitope). In this context we therefore use it here to describe the intact molecular ligand for an antibody and it should be

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properly contrasted with the term adopted by IUPAC for immunogen (Gold Book; a substance that elicits a cellular immune response and/or antibody production).

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