Ca^{2+} sensitivity of the bilayer channel varies markedly between experiments. It remains to be determined whether the channels in the patch and the bilayer are distinct, or whether the apparent differences in some of their properties result simply from differences in their membrane environment.

The bilayer channel is closed virtually all of the time in 10^{-8} M Ca^{2+} at +40 mV (Fig. 4*a*), but is open for a significant time when the Ca^{2+} concentration is raised to 10^{-6} M (Fig. 4*b*). This degree of Ca^{2+} sensitivity is in the middle of the wide range that we observed. There is no effect on channel activity after prolonged treatment (in this case 13 min) with dithionitrobenzene (DTNB)-inactivated or heat-inactivated CS (Fig. 4*c*). However, the opening and closing kinetics of the channel change dramatically within 20 s of the addition of active CS, with a large increase in open probability (Fig. 4*d*). The latency for the onset of the response ranged from too fast to measure (that is, <5 s) to as long as 7 min. The *p* values to the right of each trace in Fig. 4 demonstrate the magnitude of the probability change at +40 mV produced by phosphorylation with active CS; such changes were observed in all the experiments ($n = 11$) carried out with either the large planar bilayers or the 'tip-dip' technique. Investigation of the change in probability elicited at various voltages by CS treatment produced results (not shown) entirely consistent with a shift in channel affinity for Ca^{2+}.

Previous studies from our own and other laboratories have provided evidence that protein phosphorylation can regulate the membrane properties of excitable cells. The present results extend these findings by demonstrating directly that the activity of individual Ca^{2+}-dependent K^+ channels can be modulated by cAMP-dependent protein phosphorylation. In addition, the results put the phosphorylation site considerably closer to the channel; indeed, from the reconstitution experiments, in which individual channels are effectively at infinite dilution in the bilayer, we conclude that the phosphorylation target is either the K^+ channel itself or something intimately associated with the channel. Finally, clues have been provided about those aspects of channel function which are modulated by phosphorylation, and it will be of great interest to extend these approaches to investigate the detailed molecular mechanisms of ion-channel regulation.

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Drastic change in idiotypic but not antigen-binding specificity of an antibody by a single amino-acid substitution

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In proliferating B lymphocytes, somatic mutation of rearranged antibody variable (V)-region genes occurs at high frequency and may have a key role in the selection of these cells[1-3]. It is of interest in this context to learn in which way single mutations can affect antigen binding and/or idiotypic specificity of an antibody. Previous investigations have analysed spontaneous mutants of myeloma and hybridoma cells in which the mutation affected the antigen-binding specificity of the antibody[4-8]. Here we describe an antibody mutant that has fully retained antigen-binding specificity but has lost or drastically changed all V-region antigenic determinants (idiotopes) of the wild type as defined by monoclonal anti-idiotope antibodies. The mutant phenotype is generated by a glycine to arginine exchange in the middle of the diversity (D) element, at position 103 of the heavy chain.

The mutant was derived from the monoclonal antibody B1-8.δ1, which binds the hapten 4-hydroxy-3-nitro-5-iodophenylacetyl (NIP) with high affinity[9,10]. From a series of monoclonal anti-idiotope antibodies raised against antibody B1-8 (refs 11, 12), two were used for mutant selection. Antibody Ac38 defines an idiotope (idiotope Ac38) outside the hapten-binding cleft. In contrast, idiotope Ac146, recognized by antibody Ac146, is presumably located close to the hapten-binding site because hapten and antibody Ac146 compete for binding to the B1-8 V region. Spontaneously arising idiotope loss mutants of B1-8.δ1 were isolated by fluorescence-activated cell sorting. It has been shown previously that an $Ac146^-Ac38^+$ mutant has lost hapten-binding specificity together with the binding site-related idiotope Ac146[4]. The present experiments describe a mutant selected for the expression of the latter idiotope and against expression of idiotope Ac38.

Table 1 Affinity and fine specificity of hapten binding to wild-type and mutant antibody

	IC_{50} NIP-cap (M)	IC_{50} NP-cap (M)	Fine specificity
B1-8.δ1	1×10^{-7}	2×10^{-5}	NNP = NIP > NCLP = NP
B1-8.δ1V3	1.1×10^{-7}	1.8×10^{-5}	NNP = NIP > NCLP = NP

The IC_{50} value is the molar hapten concentration resulting in 50% inhibition of binding of antibody (60 μg ml^{-1}) to $N^{125}IP$-cap (4×10^{-8} M), determined by Farr assay as described in ref. 9. For unknown reasons, IC_{50} values for NP-cap differed reproducibly by an order of magnitude from those published previously[10]. The order of haptens according to affinity was: NNP, (4-hydroxy-3,5-dinitrophenyl)acetyl; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; NCLP, (4-hydroxy-5-chloro-3-nitrophenyl)acetyl; NP, (4-hydroxy-3-nitrophenyl)acetyl.

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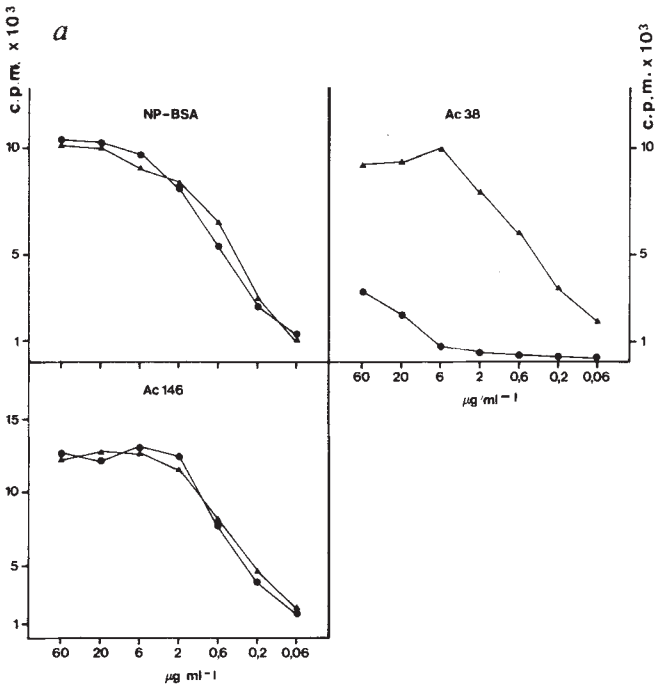

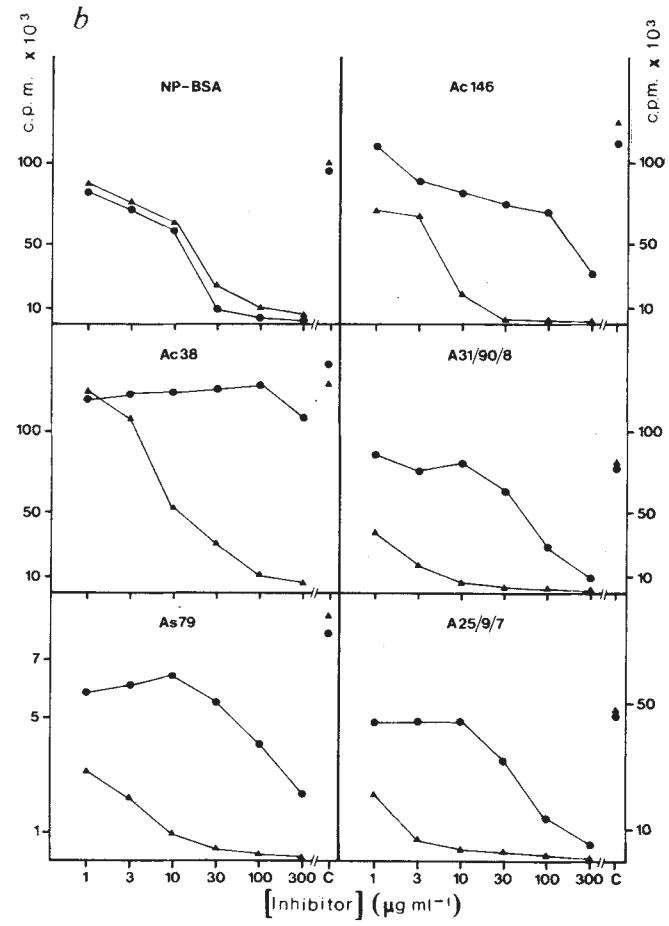

Fig. 1 Serological analysis of B1-8.δ1 (▲) and B1-8.δ1V3 (●). ***a***, Direct binding in a solid-phase radioimmunoassay[26]. Plastic plates were coated with NIP-BSA or anti-idiotope antibody at a concentration of 50 μg ml^{-1}. After saturating the plastic surface with BSA, B1-8.δ1 or B1-8.δ1V3 antibody was added to the coated wells at the concentrations indicated on the abscissa, and bound antibody detected with ^{125}I-labelled monoclonal anti-mouse λ1-chain antibody Ls136 (0.5 μg ml^{-1}; refs 11, 27). Results are shown for NIP-BSA and the anti-idiotopes Ac146 and Ac38. The anti-idiotope antibodies Ac106, As79, A25.9.7 and A31.90.8 behaved like antibody Ac38 in that they failed to bind the mutant B1-8.δ1V3 (data not shown). ***b***, Binding inhibition. The binding of iodinated antibody B1-8.δ1 to NIP-BSA or anti-idiotope-coated plates was inhibited by graded concentrations (abscissa) of unlabelled antibody B1-8.δ1 (▲) or B1-8.δ1V3 (●). Control binding without inhibitor is indicated (C).

B1-8.δ1 cells were incubated with fluoresceinated antibody Ac146 (50 μg ml^{-1}) in the presence of a 100-fold excess of unlabelled antibody Ac38. In these conditions, only cells that have retained the expression of idiotope Ac146 but lost that of idiotope Ac38 are stained. Stained cells were then enriched in eight successive rounds of sorting, expanding the separated cells after each sort. The mutant cells were finally isolated by cloning and identified by a radioimmunoassay of culture supernatant. Fifteen clones produced antibodies of mutant phenotype, that is, antibodies binding to antibody Ac146 but not to antibody Ac38. The frequency of the variant cells in the original B1-8.δ1 population is presumably $>2\times10^{-8}$, as about 5×10^{7} cells were used in the initial sorts. On the other hand, Ac146^{+}Ac38 cells were not detected in the original population by fluorescence microscopy, staining the cells simultaneously with the two anti-idiotope antibodies coupled to different fluorochromes[4] and repeatedly screening 1×10^{5} cells. These results are consistent with previous data[4] and indicate that the frequency of a given idiotype variant in B1-8.δ1 cells is at least one order of magnitude lower than the frequency of variants of the myeloma S107 which have lost the property of antigen binding.

Because of the rare occurrence of the variants and the fact that three of the variant cell clones behaved identically when tested with a variety of other anti-idiotopes, it seemed unlikely that we had isolated a series of independent mutants. Therefore, we chose only one clone, B1-8.δ1V3, for a detailed characterization. The antibody produced by this clone (antibody B1-8.δ1V3) was assayed for binding to a NIP-conjugate of bovine serum albumin (NIP_{14}-BSA) and the anti-idiotope antibodies

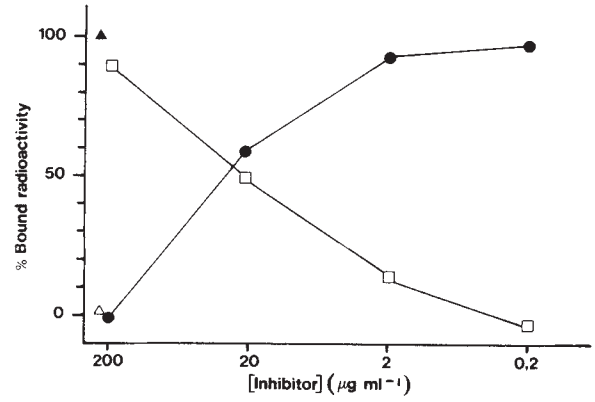

Fig. 2 Binding of λ1 light chain to variant and wild-type heavy chain. The myeloma protein HOPC1 (IgG2a, λ1) and IgD from B1-8.δ1 and B1-8.δV3 were mildly reduced with 10 mM dithioerythrol (150 min, room temperature, in the dark) and light and heavy chains separated on a Sephadex G-150 column (Pharmacia) in 1 M acetic acid with 10 mM dithioerythrol. The various fractions were dialysed first against 10 mM sodium acetate *p*H 5.5 and then against phosphate-buffered saline (PBS). When tested on a 7.5% SDS-polyacrylamide electrophoresis gel, the separated chains were >95% pure. HOPC1 λ1 chains were iodinated[27], re-associated at 20 μg ml^{-1} in PBS with 1% BSA at 4 °C overnight to either B1-8.δ1 (▲) or B1-8.δ1V3 (△) heavy chains (20 μg ml^{-1}) and tested for binding to an Ac38 (30 μg ml^{-1}) anti-idiotope-coated plastic plate. The λ1 chains can bind to the anti-idiotope only when associated with a B1-8.δ1 chain. Bound λ1 chains were counted in a γ-counter. The re-association of λ1 to B1-8.δ1 or B1-8.δ1V3 heavy chains was inhibited by increasing amounts (abscissa) of B1-8.δ1V3 (●) or B1-8.δ1 (□) heavy chains, respectively. The two curves cross each other at roughly 50% binding, indicating a similar affinity of the two heavy chains for the λ1 light chain.

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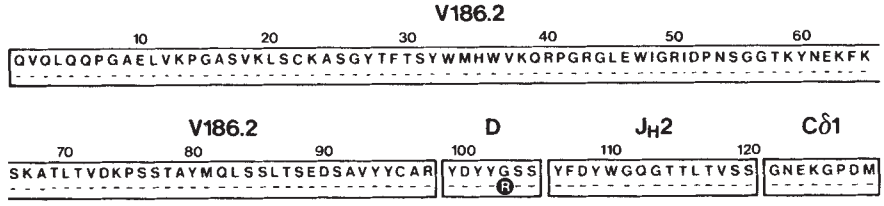

Fig. 3 Amino-acid sequence of the B1-8.δ1V3 heavy-chain variable region. The B1-8.δ1V3 antibody was cleaved by cyanogen bromide and the three fragments of the heavy-chain variable region isolated as described for B1-8.δ1 and B1-8.δ1V3 (refs 5, 28). Because H1 (residues 1–34) and H3 (residues 82–128) are blocked by the presence of N-terminal pyroglutamic acid[8], only H2 (residues 35–81) was sequenced directly by automated Edman degradation in a spinning-cup sequencer. Fragments H1, H2 and H3 were cleaved with trypsin or/and thermolysine and the fragments isolated by reverse-phase HPLC. The peptides were identified by amino-acid analysis and compared with the B1-8.δ1 sequence (upper line, single-letter code). The peptides covering residues 20–34 and 99–128 were sequenced in a gas-phase sequencer[29]. The amino-acid compositions of the peptides covering residues 1–21 and 82–98 isolated from B1-8.δ1 and B1-8.δ1V3 were indistinguishable, suggesting that for these parts both heavy chains are identical. The sequence of B1-8.δ1V3 (lower, dashed line) shows at position 103 an arginine residue instead of the glycine found for B1-8.δ1. The region of the variable regions encoded by the *D* gene (D), J_H2 gene (J_H2) and the J_H2 border to the first constant domain ($C_\delta1$) are indicated by vertical lines.

Ac146, A25.9.7, Ac38, A31.90.8, A6.24, As79 and Ac106. Efficient binding was observed only in the case of NIP-BSA and antibody Ac146 (Fig. 1*a*). The Ac146 determinant, however, is also affected by the mutation, as the B1-8.δ1V3 antibody was about 100-fold less efficient than the wild type in inhibiting the binding of radiolabelled antibody B1-8.δ1 to antibody Ac146 (Fig. 1*b*). In the case of the anti-idiotopes Ac38, A25.9.7, As79 and A31.90.8, binding inhibition by the mutant was even less efficient.

Despite its drastic idiotypic alteration, the mutant antibody inhibited the binding of the B1-8.δ1 wild type to NIP-BSA with the same efficiency as the wild-type antibody itself (Fig. 1*b*), and the affinities of both antibodies to NIP and some related haptens were identical within the limits of the analysis (Table 1).

The mutation in B1-8.δ1V3 was localized to the heavy chain. In chain re-association experiments, mutant and wild-type heavy chains, when re-associated to λ1 chains of the myeloma HOPC1, gave rise to mutant and wild-type immunoglobulin, respectively. The mutation does not interfere with the capacity of the heavy chain to bind light chain, as the two heavy chains competed with equal efficiency for re-association to λ1 (Fig. 2). Isoelectric focusing of Fv fragments revealed a small but distinct difference between the C-terminal heavy-chain variable-region fragments (relative molecular mass 19,000) of mutant and wild-type immunoglobulin (data not shown).

The amino-acid sequence of the V_H, D and joining (J) segments of the B1-8.δ1V3 heavy chain, determined by the strategy developed previously for the sequencing of B1-8.δ1V1 (ref. 5), revealed a single amino-acid difference between variant and wild type; at position 103 a glycine residue is changed to an arginine residue (Fig. 3).

In the previously isolated mutant, B1-8.δ1V1 (ref. 4), recombination (conversion) between the expressed and a non-rearranged V_H gene had generated the mutant phenotype[5,6]. Although we cannot exclude such an event in the case of B1-8.δ1V3, it seems more likely that in this antibody a point mutation has occurred, changing the glycine-encoding triplet GGT at position 103 (ref. 13) into CGT, encoding arginine. Point mutation is also the simplest explanation for the generation of mutants of the S107 myeloma line with changed antigen-binding specificity[8,14].

The observations that single amino-acid substitutions can profoundly affect idiotypic and/or antigen-binding specificity and that the D element has a key role in determining idiotypic specificity are not new[3,15–20]. The main point of the present analysis is the demonstration that a single amino-acid exchange in D can essentially eliminate the reactivity of an antibody with the majority of the anti-idiotopes raised against it, while leaving hapten-binding specificity intact. The conservation of NIP-binding specificity in the mutant, its reactivity with the NIP group on a macromolecular carrier (BSA), and the chain re-association data argue that the mutation has left the overall structure of the antibody and the hapten-binding cleft intact and selectively changed a surface structure (arginine 103 is a surface residue according to ref. 21) of key importance for idiotypic recognition. This structure may be directly involved in the interaction of the various anti-idiotope antibodies with the B1-8 V region. Like other protein interactions, idiotypic interactions seem in general to involve extended areas on the protein surface[3,19]. In the present system it has been shown that, in addition to D, residues in V_H and the V region of the light chain all contribute to the reactivity of antibody B1-8 with various anti-idiotopes[3,4,22]. It is therefore not surprising that, as far as analysed, the anti-idiotope antibodies compete with each other in binding to antibody B1-8 (ref. 11). Nevertheless, the points of contact differ for the various antibodies, as none of the target idiotopes is necessarily co-expressed with any other (refs 12, 23, 24 and our unpublished observations). Only some of the idiotopes are intimately associated with the hapten-binding site[11,12].

In biological terms, the B1-8.δV3 mutant demonstrates that a single amino-acid exchange can make an antibody essentially invisible for a variety of anti-idiotypic antibodies. Thus, somatic mutation can easily mediate escape from idiotypic control[25].

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