Complete Amino Acid Sequences of the Heavy and Light Chain Variable Regions from Two A/J Mouse Antigen Nonbinding Monoclonal Antibodies Bearing the Predominant *p*-Azophenyl Arsonate Idiotype†

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ABSTRACT: The immune response to *p*-azophenyl arsonate (Ars) in A/J mice is dominated by a cross-reactive idiotype (CRI or Id^{CR}). Id^{CR+} hybridoma proteins 1F6 and 3D10 produced in a single mouse by immunization with a monoclonal anti-Id^{CR} antibody did not bind Ars [Wysocki, L., & Sato, V. (1981) *Eur. J. Immunol. 11*, 832-839]. The preservation of idiotype coupled with lack of antigen binding in the same molecules provoked an examination of their primary structures in order to localize sites involved in binding to antigen and to anti-idiotypes. The V_H sequence of antibody 3D10 was determined by Edman degradation of intact chains and fragments generated by CNBr, hydroxylamine, and *o*-iodosobenzoic acid cleavage, by trypsin and V8 protease digestion, and by sequence analysis of mRNA. The 1F6 V_H sequence was reported previously [Smith, J. A., & Margolies, M. N. (1984) *Biochemistry 23*, 4726-4732]. The V_L sequences of 1F6 and 3D10 were determined by Edman degradation of intact chains and peptides generated by cleavage with *o*-iodosobenzoic acid and digestion with trypsin and chymotrypsin. Both 1F6 and 3D10 are encoded by the same V_H, V_κ, D, and J_κ gene segments as are Id^{CR+} Ars-binding antibodies. However, 1F6 and 3D10 employ the J_H4 gene segment rather than J_H2. Antibodies 1F6 and 3D10 share several somatic mutations, suggesting a common clonal origin, but manifest individual mutations as well. By comparison with Ars-binding Id^{CR+} molecules, the substitutions in 1F6 and 3D10 likely responsible for the lack of Ars binding are localized to the heavy chain D-J_H junction and/or to a substitution in light chain CDR 3.

Immune responses to some antigens in strains of inbred mice are dominated by antibodies that share variable-region structures that are defined serologically (idiotypes) by anti-idiotypic antibodies. Such idiotypes are phenotypic markers for germ-line genes encoding antibody variable regions and are useful in studies of antibody diversity and regulation. When A/J mice are immunized with *p*-azophenyl arsonate (Ars)[1]-protein conjugates, the spectrum of anti-Ars antibodies elaborated is dominated by a heritable cross-reactive idiotype (here designated as Id^{CR}) (Kuettner et al., 1972). Id^{CR+} immunoglobulin molecules may also be produced by means other than conventional antigen (Ars) immunization (Wysocki & Sato, 1981) and may fail to bind Ars. Immunization of A/J mice with a rat monoclonal anti-idiotypic antibody resulted in the isolation following somatic cell fusion of three idiotype-bearing hybridoma proteins that lack affinity for Ars (Wysocki & Sato, 1981). Partial amino acid sequence analyses together with DNA hybridization studies of such anti-(anti-Id^{CR}) antibodies indicated that these Ars-nonbinding (Ars^-) Id^{CR+} immunoglobulins are apparently encoded by the same V_H and V_L genes as are Ars-binding (Ars^+) Id^{CR+} immunoglobulins (Margolies et al., 1983b; Wysocki et al., 1985). The existence of homologous immunoglobulins with conserved idiotype but which fail to bind antigen prompted a search for the structural alterations responsible for failure to bind antigen. We previously reported the heavy chain variable region amino acid sequence for the Id^{CR+} Ars nonbinding hybridoma protein 1F6. We report here the complete heavy chain variable region amino acid sequence of the Id^{CR+} Ars^- hybridoma protein 3D10, as well as the complete light chain variable region sequences of both antibodies 1F6 and 3D10.

Materials and Methods

Hybridoma Cell Lines, Purification of Hybridoma Proteins, and Preparation of Heavy and Light Chains. The derivation, screening, and amplification in ascites of the cell lines 1F6 and 3D10 secreting Id^{CR+} Ars^- hybridoma proteins were described in detail previously (Wysocki & Sato, 1981; Smith & Margolies, 1984). The purification of hybridoma proteins from ascites and separation of heavy and light chains following partial reduction and alkylation of purified antibody was performed as described (Smith & Margolies, 1984).

Heavy-Chain CNBr Cleavage. Partially reduced and alkylated heavy chains were cleaved with CNBr; resultant fragments were separated by gel filtration and freed from salt as described (Smith & Margolies, 1984).

Modification of Lysine Residues and Tryptic Digestion. Heavy-chain CNBr fragments, completely reduced and alkylated light chains, and certain light-chain peptides were citraconylated (Cannon et al., 1978) or succinylated (Smith & Margolies, 1984). Trypsin digestion was performed as previously described (Cannon et al., 1978). Tryptic fragments were purified on an AcA 54 column in 5 M guanidine hy-

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[1] Abbreviations: Ars, *p*-azophenyl arsonate; CDR, complementarity-determining region; Id^{CR}, a predominant cross-reactive idiotype in the A/J strain of mouse defined by rabbit antisera; D, heavy chain diversity gene encoded region; DEAE, diethylaminoethyl; GAB, 5 M guanidine hydrochloride/0.1 M sodium acetate, pH 5.5; GTB, 5 M guanidine hydrochloride/0.1 M Tris-HCl, pH 8.5; HPLC, high-pressure liquid chromatography; IgG_1, immunoglobulin with isotope γ_1; J_H, heavy chain joining gene encoded region; OPA, *o*-phthalaldehyde; Pth, 3-phenyl-2-thiohydantoin; TFA, trifluoroacetic acid; V, variable; V_H, heavy chain variable gene encoded region; V_L, light chain variable gene encoded region; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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drochloride and 0.1 M Tris-HCl, pH 8.5, buffer (GTB). Tryptic fragments of completely reduced and alkylated, citraconylated light chains were partially purified on either AcA 202 or AcA 54 columns in 5 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5, buffer (GAB) and further purified by high-pressure liquid chromatography (HPLC) following desalting (Smith & Margolies, 1984).

Complete Reduction and Alkylation. Complete reduction and alkylation with [^{14}C]iodoacetic acid of light chains and heavy-chain fragments were performed as described (Novotny & Margolies, 1983; Juszczak & Margolies, 1983). Completely reduced and alkylated, heavy-chain CNBr and tryptic fragments therefrom were separated on AcA 54 columns equilibrated with GAB or GTB, as described (Smith & Margolies, 1984).

Iodosobenzoic Acid Cleavage. CNBr-cleaved, completely reduced and alkylated, AcA 54 purified, succinylated heavy-chain fragments or completely reduced and alkylated, succinylated light chains were cleaved with *o*-iodosobenzoic acid (Mahoney & Hermodson, 1979; Mahoney et al., 1981) as described (Juszczak & Margolies, 1983). The heavy-chain cleavage mixture was desalted on a Sephadex G-10 column in 0.03 M NH_4OH. The light-chain mixture was fractionated on an AcA 54 column in GAB and desalted on Sephadex G-10 in 0.03 M NH_4OH.

Hydroxylamine Cleavage. CNBr-cleaved, completely reduced and alkylated, AcA 54 purified, succinylated heavy-chain fragments (110 nmol) were cleaved as described (Bornstein & Balian, 1977; Steiner et al., 1979). The peptides were desalted on a Sephadex G-25 column in 9% formic acid.

Staphylococcal Protease V8 Digestion. CNBr-cleaved, completely reduced and alkylated, AcA 54 purified, succinylated heavy-chain fragments (30 nmol) were cleaved with staphylococcal protease V8 (Miles), as previously described (Houmard & Drapeau, 1972; Juszczak & Margolies, 1983). The digestion mixture was lyophilized, dissolved in 6 M guanidine hydrochloride and 0.1% trifluoroacetic acid, and purified by HPLC.

Carboxypeptidase A Digestion. CNBr-cleaved, AcA 54 purified, succinylated heavy-chain fragments were digested with carboxypeptidase A, as described (Ambler, 1972; Smith & Margolies, 1984).

Chymotrypsin Digestion. HPLC-purified light-chain tryptic fragments (20 nmol) were digested with chymotrypsin (Worthington) in 0.3 M NH_4OH and 0.1 M $CaCl_2$, pH 8.0 (0.2 mL), at 37 °C for 1.5 h at an enzyme:substrate ratio of 1:100 (w/w). The reaction was terminated by addition of 0.05 mL of glacial acetic acid. The digestion mixture was purified by HPLC.

High-Pressure Liquid Chromatography of Peptides. HPLC on μBondapak C_{18} (0.39 × 30 cm) (Waters) and RPSC C_3 (0.45 × 7.5 cm) (Beckman) columns with 0.1% trifluoroacetic acid (TFA) (Pierce) (Bennett et al., 1977) and linear gradients of acetonitrile (Baker) (1 mL/min) or isocratic acetonitrile (0.5 mL/min) were used to isolate peptides, with detection at 214 nm.

Amino Acid Analysis. Amino acid compositions were determined on a Dionex D-500 or a Beckman 6300 analyzer after hydrolysis of samples in sealed, evacuated tubes at 110 °C for 24 h in constant-boiling HCl.

Amino Acid Sequence Methods. Automated Edman degradation was performed in a Beckman 890C sequencer as described previously (Smith & Margolies, 1984; Margolies et al., 1982) or an Applied *Biosystems* 470A sequencer (Hewick et al., 1981). At cycles where proline was N-terminal, *o*-phthalaldehyde (OPA) (Pierce Chemical Co.) was added to the cup in butyl chloride from a separate reagent bottle (R4) (Spiess et al., 1983; Brauer et al., 1984). All Pth-amino acids as well as *S*-(carboxymethyl)cysteine and ε-succinyllysine were identified by HPLC on cyanopropyl columns (0.45 × 25 cm) (IBM, Danbury, CT) with 0.015 M sodium acetate, pH 5.8, containing 5% tetrahydrofuran and a linear gradient of acetonitrile (Hunkapiller & Hood, 1983).

mRNA Sequencing. Total RNA was isolated from the hybridoma cell line 3D10 according to Chirgwin et al. (1979). The mRNA encoding the expressed 3D10 heavy chain was sequenced by the dideoxynucleotide chain-termination method (Kaartinen et al., 1983; Hamlyn et al., 1978) using synthetic oligonucleotide primers that hybridize 21 bases into the constant region where the nucleotide sequences for γ1, γ2a, and γ2b are identical (Hamlyn et al., 1978). The primer was obtained from New England Biolabs.

Results

DEAE-cellulose chromatography resulted in the purification of both the 1F6 and 3D10 hybridoma proteins (yield 3–4 mg/mL of ascites) free of transferrin, the major contaminating protein eluting at low ionic strength. The purity of the hybridoma proteins was indistinguishable from that of Ars-binding monoclonal antibodies purified by affinity chromatography. The complete sequence of the 1F6 heavy chain was reported previously (Smith & Margolies, 1984).

Sequence of the 3D10 Heavy Chain. The amino acid sequence of the first 52 residues of the 3D10 heavy chain was reported previously (Margolies et al., 1983b) (Figure 1). The remainder of the variable-region protein sequence was deduced by dideoxynucleotide sequence analysis following primer extension of 3D10 heavy-chain mRNA (Hamlyn et al., 1978) except for an ambiguity in the J_H region at position 102 (Figure 2). The nucleotide sequence was confirmed by amino acid sequence analysis with methods analogous in part to those reported previously for 1F6 (Smith & Margolies, 1984), as summarized in Figure 1.

The sequence of peptide CB1-SP1 (residues 47–80, Figure 1) abruptly terminated after position 53; peptide CB1-W1 (residues 48–80) was partially sequenced with the aid of OPA blockade and overlapped the N-terminal sequence of the intact chain and peptides CB1-SP1 and CB1-SP2. However, an aspartic acid residue was identified in low yield at cycle 8 (residue 54). Following this cycle there was a marked decrease in the repetitive yield. Collectively, these data suggested that residue 54 was Asn, since the abrupt termination of Edman degradation of the CB1-SP1 peptide likely resulted from cyclization of an Asn-Gly sequence (Bornstein & Balian, 1977); in addition, identification of Asp at position 54 (cycle 8) in CB1-W1 is attributed to deamidation of the putative Asn residue at position 54. The peptide CB1-HA resulting from hydroxylamine cleavage was obtained in low yield. However, dideoxynucleotide sequencing of mRNA identified DNA codon AAT corresponding to amino acid position 54, encoding an asparagine residue (Figure 2).

Sequencing analysis of peptide CB2 revealed a single sequence for 29 cycles (residues 81–103), except for cycle 16 (residue 93). The tryptic peptides R1 and R2 were isolated from a mixture of citraconylated variable-region disulfide-linked CNBr peptides [encompassing residues 1–80 (CB1) and residues 21–80 disulfide-linked to 81–137 (CB2)] (Smith & Margolies, 1984) by gel filtration on AcA 54 before and after complete reduction and radioalkylation. The sequence of R1 (Figure 1) identifies it as originating from peptide CB2; residue 93, not identified in the sequence analysis of CB2, proved to

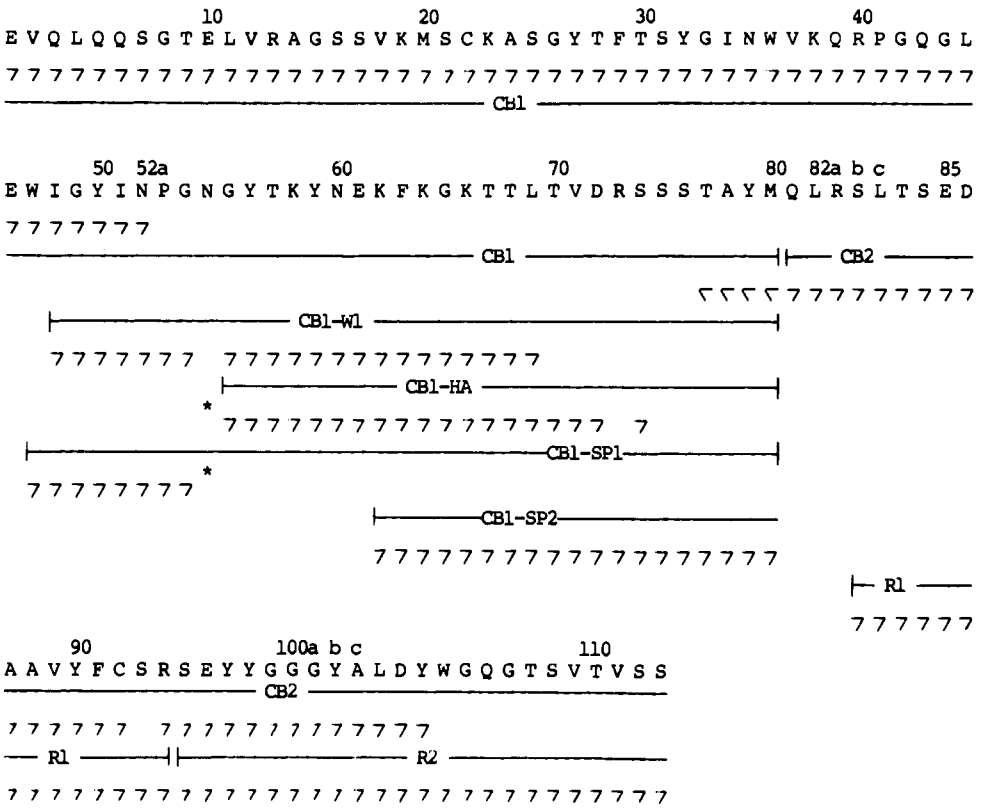

FIGURE 1: Variable-region amino acid sequence of Id^{CR+} Ars^{-} hybridoma protein heavy chain 3D10. The major peptides used to establish the sequence are indicated. CB, R, CB-W, CB-SP, and CB-HA are used to denote peptides obtained following CNBr cleavage, trypsin digestion following CNBr cleavage and citraconylation, *o*-iodosobenzoic acid cleavage of a CNBr fragment, staphylococcal protease V8 digestion of a CNBr fragment, and hydroxylamine cleavage of a CNBr fragment, respectively. Results obtained by sequence analysis of individual fragments are indicated by arrows (pointing right) below the designated fragments. Results obtained by carboxypeptidase A digestion are indicated by arrows (pointing left) below the designated fragment. Numbering is according to Kabat et al. (1983). A one-letter code for amino acids is used (IUPAC–IUB Commission on Biochemical Nomenclature, 1968).

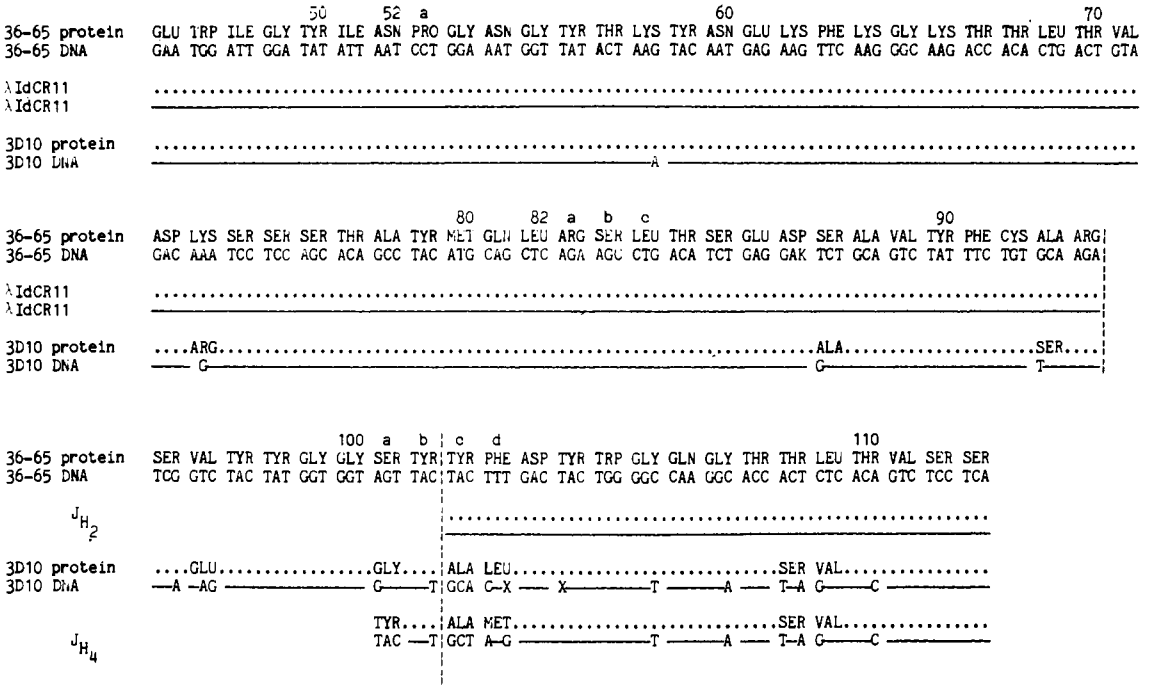

FIGURE 2: Partial amino acid and nucleotide sequences of Id^{CR+} A/J monoclonal antibody heavy chains 35–65 and 3D10. The nucleotide sequence of the V_H of the Ars-binding antibody 36–65 is identical with that of the V_H Id^{CR} germ line gene λ Id^{CR} 11, shown for comparison, in its V_H gene encoded portion (positions 1–94) (Siekevitz et al., 1982, 1983). Amino acid residues identical with those in 36–65 are indicated by a dotted line while nucleotide sequence identities are indicated by a solid line. The partial nucleotide sequence of 3D10 reported here was determined by dideoxynucleotide sequencing following primer extension of mRNA (Hamlyn et al., 1978). An X at certain positions in the 3D10 (DNA) sequence indicates ambiguity in nucleotide identification. The vertical lines indicate the V_H–D and D–J_H junctions. The nucleotide sequences and corresponding amino acid sequences of the BALB/c J_H2 and J_H4 genes are indicated for comparison (Bernard & Gough, 1980; Sakano et al., 1980). Antibody 36–65 utilizes the J_H2 gene while 3D10 utilizes J_H4. The mRNA sequence of 3D10 confirms that the amino acid residue of position 54 is asparagine (compare Figure 1). The complete V_H sequences are shown also in Figure 4.

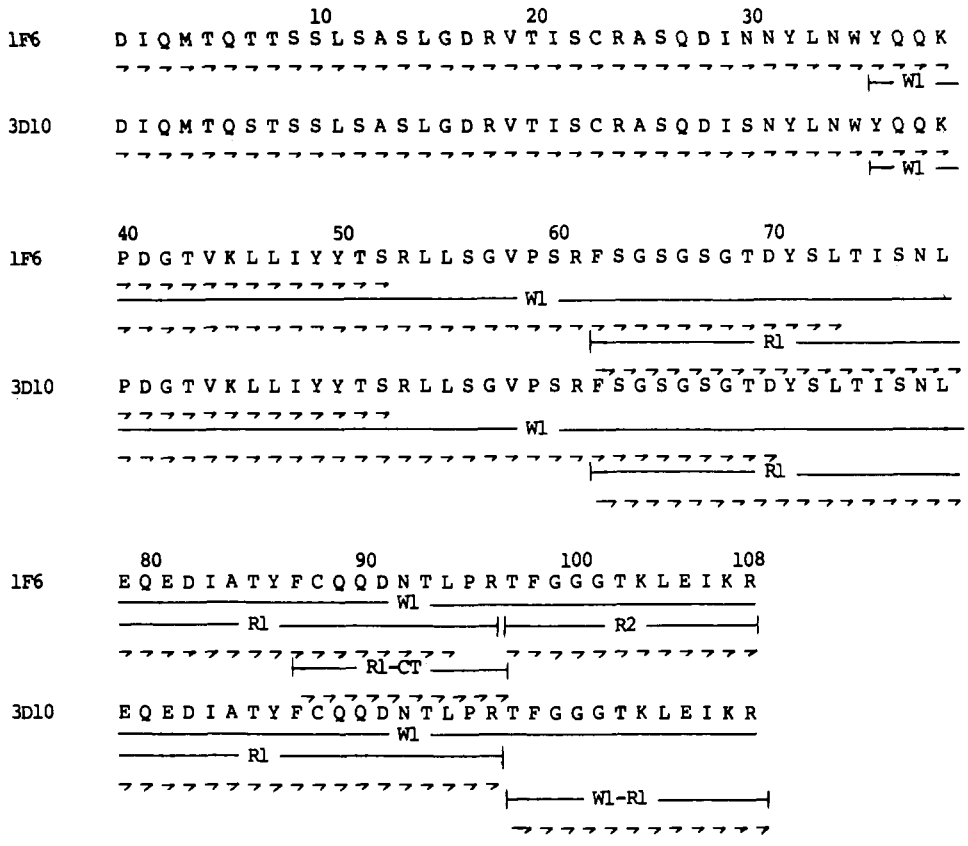

FIGURE 3: Variable-region amino acid sequences of Id^{CR+} Ars^- hybridoma protein light chains 1F6 and 3D10. The major peptides used to establish the sequence are indicated. W, R, R-CT, and W-R are used to denote peptides obtained by *o*-iodosobenzoic acid cleavage following succinylation, trypsin digestion following citraconylation, chymotrypsin digestion following trypsin digestion, and trypsin digestion following *o*-iodosobenzoic acid cleavage, respectively. Results obtained by sequence analysis of individual fragments are indicated by arrows (pointing right) below the designated fragments. Numbering is according to Kabat et al. (1983).

be serine. The sequence of peptide R2 corresponds to residues 95–137 of CB2, since the IgG_1-constant region contains a methionine at position 137 (Adetugbo et al., 1977). The amino acid sequence of the 3D10 V_H peptides (Figure 1) agrees with the partial nucleotide sequence (Figure 2).

Sequence of the 1F6 Light Chain. Sequence analysis of partially reduced and alkylated 1F6 light chains resulted in positive identification of Pth-amino acids for 52 successful cycles (Figure 3) (Margolies et al., 1983b). Because a tryptophan residue was identified at cycle 35 in the intact chain, completely reduced and alkylated 1F6 light chains were succinylated and cleaved by *o*-iodosobenzoic acid and partially purified on an AcA 54 column (Juszczak et al., 1984). Peptide W1 contained in the cleavage mixture was sequenced with OPA treatment at cycles 5 and 24 (positions 40 and 59, respectively, Figure 3). A single sequence was obtained from the mixture beginning with cycle 5 and extending for 38 cycles. This sequence (W1, Figure 3) corresponds to residues 40–73, on the basis of overlap with the sequence of the intact L chain.

To complete the 1F6 V_L sequence, completely reduced and alkylated light chains were citraconylated and digested with trypsin. Tryptic peptides were partially purified by gel filtration on AcA 54, and the peptide R1 (residues 62–96, Figure 3) was isolated therefrom by HPLC. Sequence analysis of peptide R1 provided an overlap with peptide W1, but residues 95 and 96 were not identified in this degradation. Therefore, peptide R1 was further digested with chymotrypsin, and the cleavage products were separated by HPLC. The C-terminal chymotryptic fragment of R1 (R1-CT) was identified on the basis of its radioactivity; a cysteine residue was expected at residue 88 (Figure 3). Sequence analysis of the HPLC-purified peptide R1-CT revealed a unique sequence for residues 87–96, overlapping and completing the sequence of R1.

The remaining 1F6 V_L tryptic R2 was isolated initially by gel filtration on an AcA 202 column in GAB followed by HPLC. Sequence analysis of R2 identified residues 97–108 (Figure 3), located in the sequence by homology with other known Id^{CR+} light-chain sequences (Siegelman & Capra, 1981; Ball et al., 1983) and by comparison with known murine J_κ-encoded sequences (Kabat et al., 1983).

Sequence of the 3D10 Light Chain. Edman degradation of the intact 3D10 light chain resulted in identification of the first 52 cycles (Figure 3). Because a tryptophan residue was identified at cycle 35, *o*-iodosobenzoic acid cleavage and sequence analysis using OPA blocking were carried out by methods analogous to those described for the 1F6 light chain. The sequence of peptide W1 was established for residues 40–70. An aliquot of peptide W1 was succinylated to block its N-terminus and digested with trypsin. The tryptic peptides were purified by HPLC. One of these peptides proved on Edman degradation to consist of residues 97–108 (peptide W1-R1, Figure 3).

To complete the 3D10 V_L sequence, completely reduced and alkylated light chains were citraconylated and digested with trypsin. Peptide R1 (residues 62–96, Figure 3) was isolated in a manner analogous to that described above for the corresponding 1F6 light-chain peptide R1. Sequence analysis of the 3D10 peptide R1 established residues 62–96 inclusive and provided an overlap with the W1 peptide, thus completing the sequence of the 3D10 V_L.

Discussion

Oudin and Cazenave (1971) first showed that idiotypic determinants characterizing certain antigen specificities were

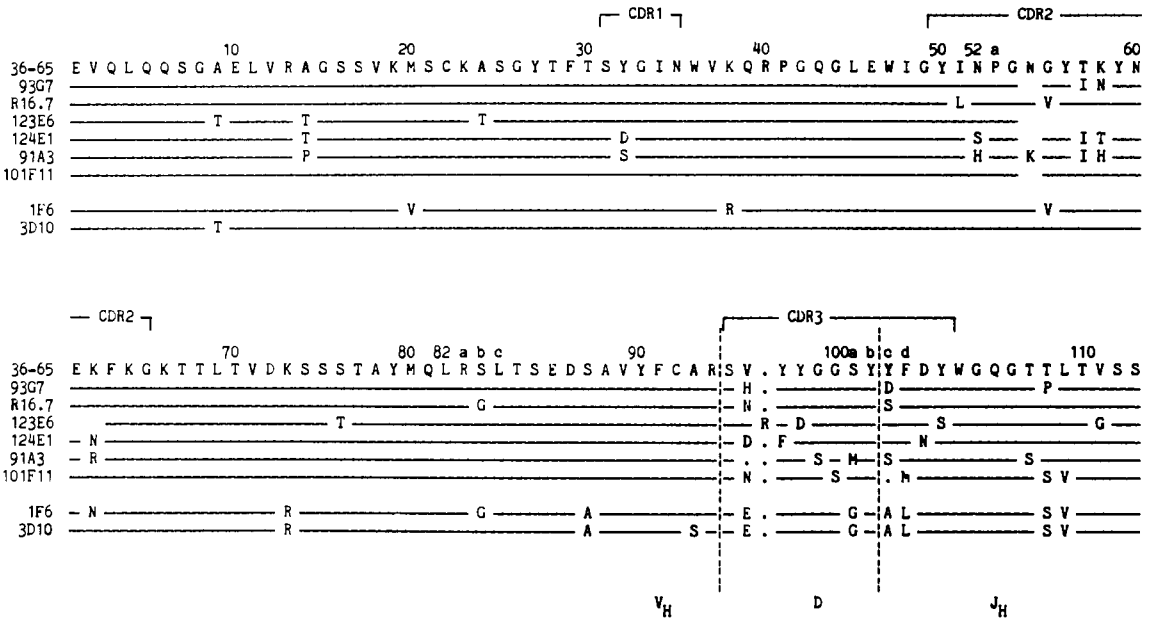

FIGURE 4: Amino acid sequences of V_H regions of A/J monoclonal antibodies. A horizontal line indicates identity with the sequence of antibody 36–65 which is identical in its V_H-encoded portion (positions 1–94) with that encoded by the germ-line gene sequence for the Id^{CR+} V_H gene λ Id^{CR}11 (Siekevitz et al., 1982, 1983). All of the antibodies shown bear the predominant Ars-associated idiotype (CRI or Id^{CR}) except for 91A3. Dots are introduced to maximize homology. Vertical broken lines indicate the V_H–D and D–J_H gene segment junctions. Numbering and designation of CDR are according to Kabat et al. (1983). The sequences of 93G7, R16.7, 123E6, 124E1, and 91A3 are from Slaughter and Capra (1983), and that of 101F11 is from Slaughter et al. (1984). The sequence of 1F6 was reported previously (Smith & Margolies, 1984); the sequence of 3D10 is reported here. All hybridoma proteins shown bind Ars except for 1F6 and 3D10; 101F11 binds Ars weakly. See also Figure 2 for comparison with certain corresponding partial nucleotide sequences.

present on some molecules that lacked specificity for that antigen, suggesting that the structural sites of idiotypy and antigen binding were distinct under certain circumstances. Examination of X-ray crystallographic structures also suggested that antigen combining sites and idiotypic determinants need not be structurally synonymous (Saul et al., 1978). In the Ars system, immunization of A/J mice with anti-idiotype resulted in expansion of both Ars^+ and Ars^- molecules bearing Id^{CR} (Wysocki & Sato, 1981). We previously demonstrated that these Ars-nonbinding Id^{CR+} molecules utilized V_H- and V_L-encoded sequences indistinguishable from those utilized by Id^{CR+} Ars^+ antibodies (Margolies et al., 1983b). Other examples of the "dissociation" of antigen binding and idiotypy are Id^{CR-} Ars^+ antibodies, which utilize the same V_H and V_L gene segments as do Id^{CR+} Ars^+ antibodies (Margolies et al., 1981, 1983a; Gridley et al., 1985), Id^{CR+} Ars-nonbinding antibodies produced by site-directed mutagenesis (Sharon et al., 1986), and other "mutant" anti-hapten antibodies (Rudikoff et al., 1982; Radbruch et al., 1985). The analysis of unique sets of antigen-nonbinding immunoglobulins bearing a predominant idiotype usually associated with a certain antigen specificity provides an opportunity to study putative idiotype–anti-idiotype regulation independent of antigen recognition. We therefore determined the complete V-region amino acid sequences of two such hybridoma proteins, 1F6 and 3D10.

The amino acid sequences of the variable region of the 1F6 and 3D10 light chains are summarized in Figure 3. The sequence of the 1F6 heavy chain was previously reported (Smith & Margolies, 1984). The amino acid sequence of the variable region of the 3D10 heavy chain and the data upon which it is based are summarized in Figure 1. A major technical problem interfering with the complete amino acid sequence analysis of peptide CB1 involved the marked decrease in yield of Pth-amino acids beginning at position 54 in peptides CB1-W1 and CB1-SP1. The tendency of asparaginyl peptides to form cyclic imides that may hydrolyze to a mixture of α- and β-aspartyl peptides is favored if glycine is the adjacent C-terminal residue (Bornstein & Balian, 1977; Steiner et al., 1979). Thus, the marked decrease in repetitive yield was attributed to a putative Asn-Gly sequence at positions 54–55. This contention was supported by the isolation of peptide CB1-HA following cleavage by hydroxylamine. Since the cleavage yield was low and aspartic acid was identified in low yield during sequencing of peptide CB1-W1, perhaps due to deamidation, it was necessary to corroborate the identity of residue 54. This was accomplished by sequence of mRNA (Hamlyn et al., 1978) (Figure 2) demonstrating the nucleotide sequence AAT at residue 54, identical with that in the V_H germ-line gene encoding Id^{CR+} molecules (Siekevitz et al., 1982, 1983). In Figure 4, the sequences of the 3D10 and 1F6 V_H regions are compared to those of six other Id^{CR+}-derived hybridoma proteins reported by Slaughter and Capra (1983) and Slaughter et al., (1984). They noted that position 54 could not be identified in hybridoma proteins 93G7, 124E1, and 101F11 (Figure 4) and was accompanied by a drop in yield on Edman degradation. However, their 93G7 cDNA *nucleotide* sequence indicated Asn at that position (Sims et al., 1982); they concluded that the asparagine was modified to preclude conventional identification. An examination of the sequences summarized in Figure 4 reveals that position 54 is not identified by amino acid sequence analysis in all chains with Gly at position 55 (93G78, 124E1, and 101F11), yet sequence analysis was unimpeded in chains where somatic mutation resulted in substitution of Lys for Asn at position 54 (91A3) or Val for Gly at position 55 (R16.7 and 1F6). These observations combined with data for the 3D10 V_H detailed herein indicate that the previous difficulties encountered in the identification of residue 54 are probably due to cyclization of the Asn-Gly sequence (54–55) during Edman degradation.

The contention that the Id^{CR+} Ars^- antibodies 1F6 and 3D10 were derived from similar or identical germ-line V_H and V_L genes with conventional Id^{CR+} Ars^+ antibodies (Margolies et

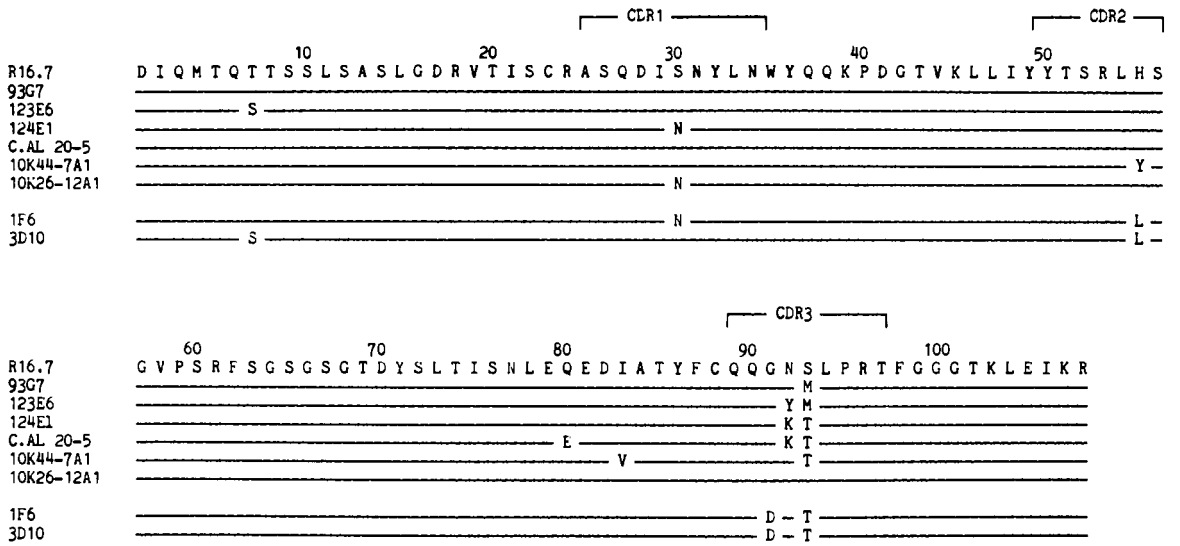

FIGURE 5: Complete V_L-region amino acid sequences from monoclonal antibodies bearing a predominant cross-reactive idiotype (Id^{CR}) associated with Ars immune response. Sequences are arbitrarily compared to R16.7. All antibodies tabulated bind Ars except for 1F6 and 3D10. All of the hybridoma proteins are from A/J mice except for CAL 20-5. The sequences of R16.7, 93G7, 123E6, and 124E1 are from Siegelman and Capra (1981); that of CAL 20.5 is from Slaughter et al. (1981); the sequences of 10K44-7A1 and 10K26-12A1 are from Ball et al. (1983); the sequences of 1F6 and 3D10 are reported here.

al., 1983b) was confirmed by Southern blot analyses for the V_H gene (Wysocki et al., 1985). In comparison to the Id^{CR} V_H germ-line gene sequence (Siekevitz et al. 1982, 1983) (residues 1–94, Figure 4), the 1F6 V_H sequence exhibits at least seven somatic mutations and the 3D10 V_H sequence four somatic mutations (Figures 2 and 4). Two substitutions (Arg-73 and Ala-87) are shared by 1F6 and 3D10. The 3D10 sequence differs also from the germ line at positions 9 (Thr) and 93 (Ser), while 1F6 contains mutations at positions 20 (Val), 38 (Arg), 55 (Val), 62 (Asp), and 82b (Gly). It is noteworthy that 1F6 and 3D10 both utilize identical D genes. The D gene utilized by most Id^{CR+} Ars^+ proteins appears similar to the BALB/c D gene $D_{FL}16.1$ (Slaughter et al., 1983, Wysocki et al., 1985; Landolfi et al., 1986). The occurrence of Gly at position 100a in both 1F6 and 3D10 as compared to other Id^{CR+} D-region sequences (Figures 2 and 4) and those reported elsewhere (Gridley et al., 1985; Landolfi et al., 1986) suggests that this substitution results from D gene somatic mutation shared by 1F6 and 3D10. Positions 96 and 100c are highly variable among Id^{CR+} V_H-region sequences, apparently the consequence of gene segment junctional diversity (Gridley et al., 1985), which affects neither the expression of idiotype nor antigen binding in the Ars system.

Ars-binding Id^{CR+} antibodies obtained following secondary antigen immunization are derived from one or a very few combinations ("canonical") of V_H, D, J_H, V_κ segments (Siekevitz et al., 1982, 1983; Slaughter & Capra, 1983; Capra et al., 1982; Siegelman & Capra, 1981; Ball et al., 1983; Slaughter et al., 1981; Wysocki et al., 1985). The A/J Id^{CR+} Ars-nonbinding molecules 1F6 and 3D10 each utilize a J_H sequence homologous to the BALB/c J_H4 gene (Sakano et al., 1980). The assignment of the J_H4 gene is supported by Southern blot analyses (Wysocki et al., 1985) and by the mRNA sequences (Figure 2), which reveal a single nucleotide difference presumed due to somatic mutation in the 3D10 J_H segment at position 100d. Both 1F6 and 3D10 also share identical V_H–D junctional residues (Figure 4).

In Figure 5, the V_L sequences of Id^{CR+} Ars^- antibodies 1F6 and 3D10 are compared to those of L chains from Id^{CR+} Ars^+ antibodies. The available sequence data suggest that, like the H chains, a single V_L gene ($V_\kappa10$) encodes Id^{CR+} L chains. Southern blot analysis also suggests that a single V_κ gene is used by most Id^{CR+} antibodies (L. Wysocki, personal communication). The 1F6 and 3D10 L chains exhibit individual substitutions at positions 7 (Ser in 3D10) and 30 (Asn in 1F6) but share identical substitutions at positions 55 (Leu), 91 (Asp), and 93 (Thr). Not only do the V_L sequences of 1F6 and 3D10 indicate a common V_L-encoded precursor, but they share identical J_κ sequences corresponding to $J_\kappa1$ of BALB/c mice (Sakano et al., 1979; Max et al., 1979; L. J. Wysocki et al., personal communication) identical with that for Id^{CR+} Ars^+ antibodies (Figure 5).

It is striking that antibodies 1F6 and 3D10 utilize all of the canonical gene segments found in Id^{CR+} Ars^+ antibodies from the secondary immune response, except for the particular J_H gene utilized. Thus, in the Ars system, monoclonal anti-(anti-idiotypic) antibodies may structurally mimic to a large extent the original idiotype. Id^{CR+} Ars^- antibodies have not been detected in secondary immune responses following Ars immunization (Pawlak & Nisonoff, 1973; Wysocki & Sato, 1981), presumably because selection is antigen-driven (Gefter et al., 1984). Thus, the ability to isolate Id^{CR+} Ars^- antibodies following anti-idiotypic immunization relates instead to selection on the basis of idiotype as the antigen, independent of Ars binding. Hybridoma proteins 1F6 and 3D10 were obtained in a fusion experiment using a single mouse immunization with a monoclonal anti-idiotype. The observations that they share the same gene segments and the same junctional residues, as well as sharing identical somatic mutations in V_H and V_L unique to these proteins, indicate that they are clonally related. The two proteins thus appear to be derived from the same B-cell precursor late in the immune response but have thereafter diverged, as each exhibits, in addition, individual somatic mutations. The clonal relatedness of monoclonal antibodies that are derived by somatic mutation over time following antigen immunization was described previously for anti-influenzal hemagglutinin antibodies (Clarke et al., 1985; McKean et al., 1984). However, the somatic mutations in antibodies 1F6 and 3D10 were not selected for by a conventional antigen (Ars) driven mechanism.

Comparison between the 1F6 and 3D10 V regions and those of Id^{CR+} Ars-binding antibodies indicates amino acid substitutions likely responsible for lack of Ars binding in 1F6 and 3D10. Certain amino acid residues among Id^{CR+} Ars^+ V

regions appear to be required for antigen binding. Siegelman and Capra (1981) suggested that the invariant V_κ–J_κ junctional Arg residue (position 96, Figure 5) was necessary for Ars binding. Further evidence was marshaled from chain recombination studies (Jeske et al., 1984). Sequence analyses of anti-Ars antibody V_L regions from a second idiotype family (Id^{36-60}) also reveal Arg at position 96, despite the use of a different V_κ gene (Juszczak et al., 1984). Structural analyses of multiple Id^{CR+} V_H regions indicate that the V_H–D junctional Ser residue at position 95 is nearly invariant in Ars-binding antibodies (Figure 4) and is not derived from either the V_H or D gene (Gridley et al., 1985; L. J. Wysocki et al., personal communication). The requirement for H-chain Ser-95 for Ars binding is supported by experiments in which this residue was changed by site-directed mutagenesis with resultant loss of Ars binding (Sharon et al., 1986). The importance of junctional diversity in determining antibody specificity has been demonstrated in other systems as well (Azuma et al., 1984) and is predicted from X-ray crystallographic studies of antibody combining sites (Poljak, 1984). Its does not necessarily follow that a light-chain junctional Arg residue and a heavy-chain junctional Ser residue are constant features of all anti-Ars antibodies irrespective of the gene segments they utilize. There may well be other steric solutions to Ars binding dictated by different primary structures. Although these residues may be necessary for Ars binding in the context of the canonical set of V-region gene segments, the results reported here indicate that binding function may be ablated by alterations elsewhere in the structure, as both 1F6 and 3D10 *do* contain these obligatory residues (Figures 4 and 5) yet fail to bind Ars.

The V_H somatic mutations occurring in 3D10 and 1F6 summarized above are unlikely to be responsible for the lack of Ars binding. Individual mutations found in each V_H (Figure 4) either are physicochemically conservative (positions 20 and 38) or are observed in Id^{CR+} Ars^+ hybridoma proteins (positions 9, 55, 62, and 82b). In addition, the two CDR 2 substitutions in 1F6 V_H are not found in 3D10, which also does not bind Ars. Although the Ser-93 substitution in the 3D10 V_H might conceivably alter the conformation of the adjacent CDR 3, a different hypothesis would be required to explain the failure of Ars binding in 1F6. It is more likely that the binding defect in the two proteins is shared and occured in a common clonal precursor prior to further individual somatic mutation. The common Ala substitution at heavy-chain position 87 is a candidate; however, on the basis of comparison with the McPC 603 structure (Segal et al., 1974), this framework residue is spatially removed from the antigen binding site.

With respect to the V_H–D junction (Figure 4), the glutamic acid residue at position 96 in 1F6 and 3D10 is also seen in the Id^{CR+} Ars^+ antibodies 36–71 and 44–10 (data not shown; Gridley et al., 1985) and is thus unlikely to affect Ars binding. The somatic mutation in the D gene segment at position 100a (Gly in 1F6 and 3D10) could affect Ars binding. However, the Id^{CR+} Ars^+ antibodies 45–223 (Gridley et al., 1985; data not shown) and the Id^{CR-} Ars^+ antibody 91A3 (Figure 4; Slaughter & Capra, 1983) also exhibit "mutations" at this position. Among certain anti-Ars antibodies employing canonical V_H, J_H, V_κ, and J_κ gene segments, extensive variability of the core D gene sequence occurs (Figure 4; Gridley et al., 1985), yet Ars binding is preserved. On the other hand, in most instances, expression of Id^{CR} determinants requires conservation of the core D gene sequence (Gridley et al., 1985; Meek et al., 1984). Since antibodies 1F6 and 3D10 utilize V_H, D, $V\kappa$, and J_κ segments identical with those in Id^{CR+} Ars^+ antibodies but utilize the J_H4 gene segment instead of J_H2, it was tempting to assign the lack of Ars affinity to the use of J_H4. In mutant anti-phosphocholine antibodies dramatic changes in antigen binding properties were due to a single J_H amino acid difference (Cook et al., 1982). However, Id^{CR+} Ars^+ antibodies that employ *unmutated* J_H4 gene segments (assuming A/J and BALB/c have identical J_H4 sequences) were isolated from primary immune responses (Wysocki et al., 1985), indicating that the use of J_H4 per se and the resultant fixed amino acid sequence differences between J_H4 and J_H2 at positions 108 and 109 (Figure 4) cannot account for the lack of Ars binding in 1F6 and 3D10. Decreased Ars affinity in the Id^{CR+} antibody 101F11 (Figure 4; Slaughter et al., 1984) may be related to the use of J_H4. That protein has an unmutated V_H sequence and a deletion of the first three nucleotides of the J_H4 gene, rendering it one residue shorter than the other antibodies depicted in Figure 4. As noted above, position 100c at the D–J_H junction exhibits a remarkable degree of variability, with at least five different residues at that position [Figure 4 and data from Gridley et al. (1985)] despite preservation of Ars binding. At both positions 96 and 100c, hydrophobic, acidic, and basic amino acids occur without apparent gross disturbance of Ars binding in the context of the canonical gene segment combination. Antibodies 1F6 and 3D10 reported here do not contain a deletion in the J_H, but both exhibit a somatic mutation at position 100d (Leu) in J_H4, which is indeed a candidate for ablation of antigen binding function.

Light-chain sequence differences in 1F6 and 3D10 may also account for absent Ars binding. The individual substitutions at positions 7 in the framework and 30 in the CDR 1 are seen in Id^{CR+} Ars^+ molecules and can thus be excluded (Figure 5). Similarly, the shared Thr residues at position 93 are also seen in several other light chains from Ars^+ antibodies. The leucine residues at position 55 in both 1F6 and 3D10 represent a unique substitution in comparison to other Ars^+ V_L sequences (Figure 5). Most light chains bear His at this position (the germ-line sequence is not yet known). However, variability in this V_L CDR 2 locus is permitted with preservation of antigen binding as manifested by the use of Tyr in antibody 10K26-12A1 (Figure 5), Gln in antibody 36-71, and Lys in antibody 45-49 (M. Margolies, unpublished results). The unique Asp substitution at position 91 in 1F6 and 3D10 rather than Gly observed in all other Id^{CR+} Ars^+ V_L sequences is as likely a source of loss of specific Ars binding as the J_H4 somatic mutation described above. Further precise assignment of the structural "defects" in 1F6 and 3D10 will require correlation with three-dimensional structures as determined by X-ray crystallography. The successful crystallization of Id^{CR+} Ars^+ antibodies has been reported (Amit et al., 1983; Rose et al., 1986). Additional evidence bearing on this point is likely to come from experiments employing site-directed mutagenesis and sequence analysis of Ars-binding antibodies, which utilize J_H4, that are elaborated in the primary immune response (Wysocki et al., 1985) and are expected to be free of somatic mutations.

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SUPPLEMENTARY MATERIAL AVAILABLE

Eight figures showing elution profiles of mixtures of tryptic

peptides (R1 and R2) from 3D10 heavy chains and 1F6 light chains, HPLC separation patterns for CNBr peptides (CB2) and staphylococcal protease V8 peptides (CB1-SP1 and CB1-SP2) from 3D10 heavy chains, and tryptic and chymotryptic peptides (R1, R2, and R1-CT) from 1F6 light chains (8 pages). Ordering information is given on any current masthead page.

Registry No. *p*-Azophenyl arsonate, 7334-23-8.

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Kinetic Analysis of Guanosine 5′-*O*-(3-Thiotriphosphate) Effects on Phosphatidylinositol Turnover in NRK Cell Homogenates†

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ABSTRACT: Addition of the guanine nucleotide analogue guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) to [^{3}H]inositol-labeled NRK cell homogenates resulted in rapid breakdown of cellular polyphosphoinositides. GTPγS stimulated phospholipase C, resulting in a more than 4-fold increase in the hydrolysis rates of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bis(phosphate) (PIP_2). No significant effect of GTPγS on direct phosphatidylinositol (PI) hydrolysis was detected. There was an increase in water-soluble inositols, with inositol tris(phosphate) (IP_3) levels increasing at least 10 times over the decrease seen in PIP_2, indicating that PIP kinase activity was also accelerated following GTPγS addition. Inositol 1,4,5-tris(phosphate) peaked rapidly after GTPγS addition (less than 2 min) while inositol 1,3,4-tris(phosphate) was produced more slowly and leveled off after approximately 10 min. The differential equations describing conversion between intermediates in the PI turnover pathway were solved and fitted to data obtained from both [^{3}H]inositol and [^{32}P]phosphate fluxes by nonlinear least-squares analysis. GTPγS effects on the pseudo-first-order rate constants for the lipase, kinase, and phosphatase steps were determined from the analysis. From these measurements it can be estimated that, in the presence of GTPγS and calcium buffered to 130 nM, hydrolysis of PIP_2 accounts for at least 10 times as much diacylglycerol as direct PI breakdown despite the 100-fold excess of PI over PIP_2. From the kinetic model it is predicted that small changes in the activities of PI and PIP kinases can have large but different effects on the level of IP_3 and diacylglycerol following GTPγS addition. These results argue that regulation of PI and PIP kinases may be important for determining both cellular IP_3 and diacylglycerol levels.

Stimulation of cells by a variety of hormones and mitogens activates a transducing mechanism that involves phosphoinositide breakdown (Berridge & Irvine, 1984; Macara, 1985; Whitman et al., 1986a). The key reaction of this transducing mechanism is a receptor-mediated hydrolysis of phosphatidylinositol 4,5-bis(phosphate) (PIP_2)[1] to give diacylglycerol (DG) and inositol tris(phosphate) (IP_3) (Berridge, 1984). IP_3 and DG have both been shown to act as second messengers, IP_3 by mobilizing intracellular Ca^{2+} (Streb et al., 1983) and DG by stimulating protein kinase C (Nishizuka, 1984). Although much of the study of PI turnover has been in terminally differentiated and nonproliferating cells that show responses to specific hormones, it has been argued that this system originally evolved for regulation of cell growth and was later adapted for specific tissue responses (Macara, 1985; Whitman et al., 1986a). A role for PI turnover in normal cell proliferation has been suggested by the ability of Ca^{2+} ionophore A23187 and specific activators of protein kinase C such as phorbol esters to mimic or enhance early effects of growth factors on quiescent cells (Mastro & Smith, 1983). Tumorigenesis is associated with the uncontrolled proliferation of cells, and specific oncogenes have been implicated in this process (Bishop, 1985). Cells transformed by several types of oncogenes have accelerated PI turnover compared with nontransformed cells, suggesting that enhanced PI turnover may be a critical part of the transformation mechanism (Diringer & Friis, 1977; Sugimoto et al., 1984; Macara et al., 1984; Fleischman et al., 1986; Kaplan et al., 1986).

The mechanism by which hormones, mitogens, and certain oncogene products activate PI turnover is unknown. The lack

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[1] Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bis(phosphate); IP_1, inositol 1-phosphate; IP_2, inositol 1,4-bis(phosphate); IP_3, inositol 1,4,5-tris(phosphate) + inositol 1,3,4-tris(phosphate); Ins-1,4,5-P_3 or IP_3(1,4,5), inositol 1,4,5-tris(phosphate); Ins-1,3,4-P_3 or IP_3(1,3,4), inositol 1,3,4-tris(phosphate); Ins-1,3,4,5-P_4 or IP_4(1,3,4,5), inositol 1,3,4,5-tetrakis(phosphate); ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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