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Immunology

Efficient generation of antibodies to oncoproteins by using synthetic peptide antigens

(oncogenes/transformed cells)

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ABSTRACT **To examine the efficiency of generating protein-reactive antipeptide antibodies, 35 peptides encoded by retroviral or cellular oncogenes were used to immunize rabbits. Thirty-two peptides elicited antipeptide antibodies, of which 56% reacted with their respective oncoproteins. The length of the immunizing peptide was an important factor in generating antibodies reactive with native protein. Similar peptides differing in a single or a few amino acids could elicite antisera of markedly different reactivities.**

Antibodies generated by use of chemically synthesized peptides as immunogens have proved useful in detecting proteins predicted from nucleic acid sequences in cells and in studying functionally active regions of proteins and in following the fate of protein domains (1). Recent advances in the technology of sequencing nucleic acids have enhanced the potential usefulness of peptide antibodies and a large number of different protein-reactive antipeptide antibodies have been generated (2–6).

There is little doubt about the usefulness of the antipeptide antibody technique, however, some practical problems are still to be resolved prior to its widespread application. In some studies the success rate is as low as 25%, whereas with other peptides it is as high as 100% (7). In establishing the immunological basis of this approach to antibody generation, it is useful to define the characteristics of the peptide necessary for eliciting protein reactive antibodies and to analyze the reactivities of the antibodies to both peptides and native proteins. To examine the efficiency of this system in generating protein-reactive antibodies utilizing synthetic peptides, we chose peptide sequences encoded in retroviral and cellular oncogenes. In many cases the complete nucleic acid sequences of these genes are available and the oncogene products are biologically important.

MATERIALS AND METHODS

Cell Lines. Cell lines used in this study as sources of oncoproteins were as follows: BM2 cells (avian myeloblastosis virus-transformed chicken myeloid cells containing v-*myb*), normal rat kidney cells (NRK), NRK cells transformed by Harvey sarcoma virus (NRK-H containing v-Ha-*ras*), NRK transformed by Kirsten sarcoma virus (NRK-K containing v-Ki-*ras*), BALB 3T3, Rous-sarcoma virus transformed 3T3 (3T3^{src} containing v-*src*), Snyder–Theilen feline sarcoma virus (ST-FeSV)-transformed mink and cat cells (B3TI and 82C containing v-*fes*), and avian myelocytomatosis virus-transformed chicken cells (MC29 containing v-*myc*). The cell lines were generously provided by S. Aaronson, M. Essex, M. Baluda, and P. Duesberg.

Peptides and Immunizations. Peptides were synthesized according to predicted amino acid sequences based on nucleotide sequences of c- and v-*src* (8, 9), v- and c-Ha-*ras* (10, 11), v-Ki-*ras* (12), v-*myb* (13), v-*fes* (14), and v-*myc* (15). Selection of the peptide regions for synthesis were based on hydrophilicity indices determined by the method of Hopp and Woods (16). The peptides used in this study were synthesized by Peninsula Laboratories (Belmont, CA). Purity of individual peptides was confirmed by HPLC.

Peptides were coupled to keyhole limpet hemocyanin (6) and 300 μg of conjugates was injected subcutaneously in complete Freund's adjuvant into New Zealand White rabbits. Several rabbits were immunized with peptides of special interest. Four weeks later, rabbits were injected with 175 μg of conjugate in incomplete Freund's adjuvant. Injections were repeated with conjugated or unconjugated peptides at intervals varying between 2 weeks and 4 months. Rabbits were bled 10 days after each injection.

Antibody titers against peptide were determined by an enzyme-linked immunosorbent assay (ELISA) (17). Briefly, microtiter plates (Immulon 2, Dynatech, Alexandria, VA) were coated with peptide solution [2 μg of peptide in 100 μl of 50 mM carbonate buffer (pH 9.5) per well] for 6 hr. After washing out unbound peptide with 0.02% Tween 20 in phosphate-buffered saline (P_i/NaCl) overnight, plates were incubated with blocking solution (3% bovine serum albumin in P_i/NaCl) overnight to prevent nonspecific binding of antiserum. Antiserum dilutions of 10^{-1} to 10^{-5} in 0.1% bovine serum albumin in P_i/NaCl were added and incubated for 1 hr. Antibody binding was detected by peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Binding was visualized by addition of substrate solution [40 μg of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) and 0.03% H_2O_2 in 100 μl of 0.1 M citrate buffer, pH 4.5]. The titer of the antiserum was taken to be the maximal dilution at which the ELISA reaction could be visualized. Preimmune serum from each rabbit served as a negative control.

Reactions of antiserum with oncoprotein were determined by immunoprecipitation (18) or immunoblotting (19). Cells were radioactively labeled with [^{35}S]methionine (Amersham; specific activity, 1.105 Ci/mmol; 1 Ci = 37 GBq), washed three times with P_i/NaCl and lysed at a concentration of 10^7 cells per ml of lysis buffer (1% Triton X-100/0.01% sodium azide/0.5% sodium deoxycholate/0.1% NaDodSO$_4$/0.1 M sodium chloride/5 mM EDTA/0.4 trypsin inhibitory unit/ml of aprotinin/0.1 mM phenylmethylsulfonyl fluoride in 0.1 M phosphate buffer, pH 7.5), and then centrifuged at 100,000 × *g* for 30 min at 4°C. Preimmune serum (10 μl) was mixed with 500 μl of labeled cell lysate (3–4 × 10^6 cpm). After overnight incubation on ice, the complexes were absorbed by 100 μl of Pansorbin [10% suspension of *Staphylococcus aureus* bearing protein A (Calbiochem)] for 2 hr. After centrifugation, the

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supernatant was incubated with 10 μl of antipeptide antiserum and reprecipitated. The *S. aureus* cells bearing immune complexes were washed twice with the lysis buffer and once with 0.5 M lithium chloride in 0.1 M Tris Cl buffer (pH 8.0). The immune complexes were eluted in 50 μl of elution buffer (7.5% $NaDodSO_4$/2.5% 2-mercaptoethanol/100 mM dithiothreitol/1 mM EDTA/2 M urea in 0.1 M Tris Cl buffer, 6.8) for 40 min.

After centrifugation, the supernatant was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in a discontinuous buffer system. The gel was fixed in 50% methanol/10% glacial acetic acid then processed for fluorography (20) and exposed to Kodak x-ray film in conjunction with Dupont Cronex intensifying screens.

For immunoblotting, cell lysates (2–3 × 10^7/ml) were prepared in lysis buffer without $NaDodSO_4$. The protein concentration of a supernate (12,000 × *g*, 30 min) was determined (21). Volumes equivalent to 50 μg and 40 μg of protein were loaded on the 15- and 20-well $NaDodSO_4$-polyacrylamide gels, respectively. After electrophoresis, the gels were placed in a multilayer "sandwich" of nitrocellulose filters, 0.45-μm pore size, and placed in a chamber containing 20 mM Tris/150 mM glycine/20% (vol/vol) methanol. Electrolytic transfer was carried out toward the anode at 45 V (0.10–0.12 A) overnight. Residual binding sites on the filters were blocked by incubation with 2.5% bovine serum albumin in 50 mM Tris Cl/0.85% NaCl, pH 7.4 (Tris/saline), for 30 min. The filters were incubated with the appropriate antiserum (diluted 1:20–25) overnight at 4°C on a rocking platform, washed twice with 0.05% Nonidet P-40 in Tris/saline and twice with Tris/saline over 40 min, then incubated with ^{125}I-labeled protein A (New England Nuclear, 0.5–3 × 10^6 cpm in 50 ml of 2.5% bovine serum albumin in Tris/saline) for 1 hr. After washing thrice with 0.05% Nonidet P-40 in Tris/saline, thrice with Tris/saline, and once with distilled water, the filters were dried and exposed to Kodak x-ray film in conjunction with intensifying screens.

RESULTS

Production of Antipeptide Antisera. Thirty-five different peptides were used to immunize rabbits and the serum titers obtained after a second injection of peptide antigen are shown in Table 1. Twenty-nine rabbits (83%) responded and gener-

Table 1. Peptides synthesized and reactivities of antipeptide antisera

Peptide	Oncogene	Length, no. amino acids	Position	Hydrophilic index	Amino acid sequence	Reactivity of antiserum	
						Peptide*	Protein†
1	c-*src*	17	409–425	+0.50	R-L-I-E-D-N-E-Y-T-A-R-Q-G-A-K-F-P	10^4	+
2	c-*src*	15	468–482	+0.66	N-R-E-V-L-D-Q-V-E-R-G-Y-R-M-P	10^4	–
3	c-*src*	10	499–508	+1.42	W-R-R-D-P-E-E-R-P-T	10^4	+
4	v-Ki-*ras*	18	1–18	−0.17	M-T-E-Y-K-L-V-V-V-G-A-S-G-V-G-K-S-A	10^5	++
5	v-Ki-*ras*	17	119–135	+0.52	D-L-P-S-R-T-V-D-T-K-Q-A-Q-E-L-A-R	10^5	++
6	v-Ki-*ras*	15	161–175	+1.32	R-E-I-R-Q-Y-R-L-K-K-I-S-K-E-E	10^2	–
7	v-Ki-*ras*	19	167–185	+1.15	R-L-K-K-I-S-K-E-E-K-T-P-G-C-V-K-I-K-K	10^3	++
8	v-Ha-*ras*	18	1–18	−0.01	M-T-E-Y-K-L-V-V-V-G-A-R-G-V-G-K-S-A	10^4	–
9	c-Ha(EJ)-*ras*	18	1–18	−0.26	M-T-E-Y-K-L-V-V-V-G-A-V-G-V-G-K-S-A	10^3	–
10	c-Ha-*ras*	18	1–18	−0.17	M-T-E-Y-K-L-V-V-V-G-A-G-G-V-G-K-S-A	10^5	++
11	v-Ha-*ras*	16	29–44	+0.73	V-D-E-Y-D-P-T-I-E-D-S-Y-R-K-Q-V	10^4	+++
12	v-Ha-*ras*	18	91–108	+1.26	E-D-I-H-Q-Y-R-E-Q-I-K-R-V-K-D-S-D-D	10^4	++
13	v-Ha-*ras*	11	126–136	+0.67	E-S-R-Q-A-Q-A-L-A-R-S	10^4	–
14	v-Ha-*ras*	10	146–155	+0.93	A-K-T-R-Q-G-V-E-D-A	10^5	–
15	v-Ha-*ras*	20	160–179	+0.86	V-R-E-I-R-Q-H-K-L-R-K-L-N-P-P-D-E-S-G-P	10^5	++
16	v-*myb*	14	11–24	+0.58	P-Q-E-S-S-K-A-G-P-P-S-G-T-T	10^4	–
17	v-*myb*	15	33–47	−0.52	M-A-F-A-H-N-P-P-A-G-P-L-P-G-A	10^3	+
18	v-*myb*	17	146–162	+0.27	D-N-T-R-T-S-G-D-N-A-P-V-S-C-L-G-E	10^4	++
19	v-*myb*	19	168–186	+0.22	P-S-P-P-V-D-H-G-C-L-P-E-E-S-A-S-P-A-R	10^4	–
20	v-*myb*	16	170–185	+0.23	P-P-V-D-H-G-C-L-P-E-E-S-A-S-P-A	10^2	–
21	v-*myb*	14	247–260	+0.37	P-F-H-K-D-Q-T-F-T-E-Y-R-K-M	10^4	+
22	v-*myb*	19	247–265	+0.14	P-F-H-K-D-Q-T-F-T-E-Y-R-K-M-H-G-G-A-V	10^4	++
23	v-*fes*	15	541–555	+1.15	R-H-S-T-S-S-S-E-Q-E-R-E-G-G-R	10^4	+++
24	v-*fes*	10	584–593	+0.56	P-E-V-Q-K-P-L-H-E-Q	10^4	++
25	v-*fes*	15	782–796	+1.80	F-L-R-T-E-G-A-R-L-R-M-K-T-L-L	10^4	–
26	v-*fes*	7	840–846	+1.18	S-R-E-A-A-D-G	(−)	ND
27	v-*fes*	13	893–905	−0.06	A-S-P-Y-P-N-L-S-N-Q-Q-T-R	10^3	++
28	v-*fes*	13	901–913	+0.86	N-Q-Q-T-R-E-F-V-E-K-G-G-R	10^4	+++
29	v-*myc*	13	222–234	+1.17	P-P-T-T-S-S-D-S-E-E-E-Q-E	(−)	ND
30	v-*myc*	12	323–334	+1.45	R-T-L-D-S-E-E-N-D-K-R-R	10^4	–
31	v-*myc*	11	340–350	+1.61	E-R-Q-R-R-N-E-L-K-L-R	10^4	–
32	v-*myc*	9	363–371	+0.65	N-N-E-K-A-P-K-V-V	10^1	–
33	v-*myc*	15	389–403	+1.43	R-L-I-A-E-K-E-Q-L-R-R-R-R-E-Q	10^4	++
34	v-*myc*	11	395–405	+1.61	E-Q-L-R-R-R-R-E-Q-L-K	10^4	–
35	v-*myc*	7	400–406	+1.41	R-R-E-Q-L-K-H	(−)	ND

Abbreviations: G, glycine; A, alanine; V, valine; L, leucine; I, isoleucine; F, phenylalanine; P, proline; M, methionine; S, serine; T, threonine; Y, tyrosine; W, tryptophan; N, asparagine; Q, glutamine; C, cysteine; D, asparatic acid; E, glutamic acid; K, lysine; R, arginine; H, histidine.
*ELISA: The maximal dilution in which the reaction could be visualized was used as the titer of the antipeptide antiserum.
†Reactivity of antisera against the following oncoproteins was examined in cell lysates by immunoprecipitation and/or immunoblotting: pp60 (*src*), p21 (Ki-*ras* and Ha-*ras*), p48 (*myb*), p85 (*fes*), p110 (*myc*). The reactivity of antisera to oncoproteins was defined arbitrarily as strong (+++), fair (++), weak (+), or negative (−) as based on the radioautographs performed under standardized conditions. ND, not done.

ated antipeptide antibody with titers $\geq 1:10^3$. A lower antibody response was obtained to peptides 6, 20, and 32. The titer increased from $1:10^2$ to $1:10^4$ with subsequent injections of peptide 6 but not of peptide 20 or 32. Three peptides 26, 29, and 35 failed to induce any antibody responses in the injected animals. It was evident that the length of the peptides used as antigen was important for antibody production. All three peptides with fewer than 10 amino acid residues failed to induce effective antibody responses (Fig. 1). On the other hand, 31 of 32 peptides (97%) with more than 10 amino acid residues induced high titers of antibodies. No correlation between the hydrophilicity of the peptide (within the range studied) and antibody titer was observed (Fig. 2).

Reactivity of Antipeptide Antiserum with Native Protein. The 32 antipeptide antisera generated in this study were tested by immunoblotting or immunoprecipitation for their ability to react with their respective oncoproteins derived from established retrovirus-transformed cell lines. The target proteins were as follows: 60-kDa protein ($pp60^{src}$) for v-*src* in $3T3^{src}$ cells; 21-kDa proteins ($p21^{Ha\text{-}ras}$ or $p21^{Ki\text{-}ras}$) for v-Ha-*ras* and v-Ki-*ras* in NRK-H and NRK-K cells, respectively; 48-kDa protein ($p48^{myb}$) for v-*myb* in NP cells; 85-kDa protein ($p85^{gag\text{-}fes\text{-}GT}$) for v-*fes* in 82C and/or B3TI cells; and 110-kDa protein ($p110^{gag\text{-}myc}$) for v-*myc* in MC29 cells. Controls for all experiments included preimmune serum as well as competitive inhibition of reactions between antibody and oncoprotein by the appropriate peptide. Eighteen of 32 antipeptide antisera (56%) reacted with their respective oncoproteins, although the efficiency varied from 20% for anti-*myc* peptides to 80% for anti-*fes* peptides (Table 1). The reactivity of the antisera against oncoproteins was independent of the position of the peptide in the native molecules (Fig. 3). No correlation was observed between reactivity with native protein and the hydrophilicity of the peptide in the range of -0.52 to $+1.80$ (Fig. 4). Peptides of fewer than 12 amino acids were relatively unsatisfactory for generating antibodies that reacted strongly with oncoproteins. Only one out of seven such peptides generated a moderately reactive antiserum (Fig. 5).

Effect of Amino Acid Substitutions on Specificity of Antibody. Four ras peptides of residues 1–18 (peptides 4, 8, 9, 10), differing in only a single amino acid at position 12 (Table 2), induced antisera with titers greater than $1:10^3$. Each antipeptide antiserum cross-reacted with each of the other three peptides but reaction to the oncoproteins varied. Antiserum to v-Ki-*ras* peptide (peptide 4) reacted strongly with p21 oncoproteins in both NRK-K and NRK-H cells. Antiserum raised against peptide 10 (c-Ha-*ras*) reacted with p21 in NRK-H but not in NRK-K cells. The antisera to peptides 8 and 9 (v-Ha-*ras* and c-Ha(EJ)-*ras*) were unreactive with any *ras* oncoprotein. Thus, a single change among the 18

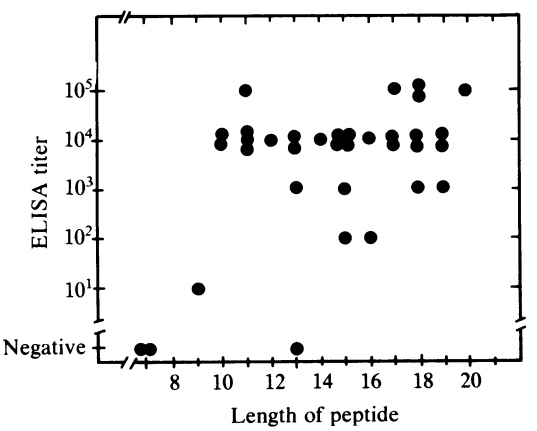

FIG. 1. Effect of the length of the peptides (number of amino acids) used as immunogens on the production of antibodies.

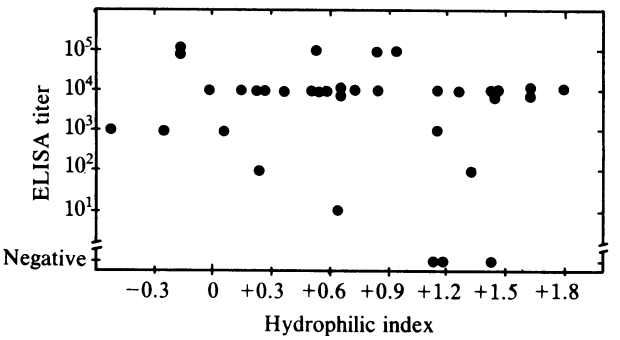

FIG. 2. Effect of the hydrophilicity of the immunizing peptides on antibody production.

amino acids of the peptide sequence was sufficient to produce a significant effect on the specificity of the antiserum for the oncoproteins.

In addition to the ras peptides, we examined four pairs of peptides that shared common amino acid sequences: peptides 33 and 34 (v-*myc*, residues 389–403 and 395–405), peptides 27 and 28 (v-*fes* residues, 893–905 and 901–913), peptides 19 and 20, (v-*myb*, residues 168–186 and 170–185), and peptides 21 and 22 (v-*myb*, residues 247–260 and 247–265) as shown in Table 2. All of these peptides induced antibodies reactive with the immunizing peptides. Despite a common nine amino-acid sequence in the peptides, the two anti-*myc*-peptide antisera did not cross-react with the heterologous peptide. Antiserum to peptide 33 recognized the v-*myc* oncoprotein, $p110^{gag\text{-}myc}$, but antiserum to peptide 34 did not. The two peptides, 27 and 28 of v-*fes*, shared a common sequence of five amino acids among 13 residues. Antisera to these peptides did not cross-react with the heterologous peptide but both detected the v-*fes* oncoprotein, $p85^{gag\text{-}fes\text{-}GT}$. Anti-*myb* antisera against peptides 19 and 20 were cross-reactive with the heterologous peptides while neither antiserum reacted with the $p48^{myb}$ oncoprotein. On the other hand, antisera to peptides 21 and 22 cross-reacted with heterologous peptide and each reacted with the $p48^{myb}$ oncoprotein.

These findings suggest that the immunogenic domains of peptides differ even when they have relatively long sequences in common and that the immunogenic regions of peptides are often dissimilar from the antigenic regions of the homologous native protein molecules.

DISCUSSION

The production of antibodies to synthetic peptides is a versatile tool in the molecular analysis of proteins (1, 2, 7, 22) but there are still some questions about the design of peptides

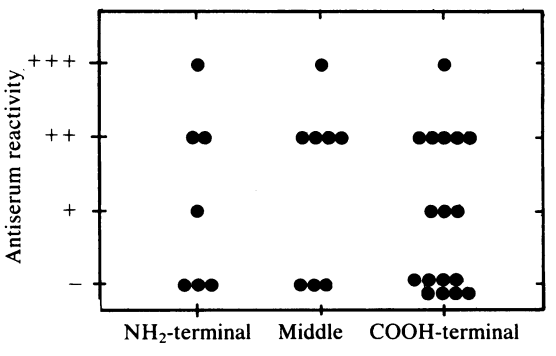

FIG. 3. Effect of the position of the immunogenic peptides in the oncoproteins upon the reactivity of antipeptide antisera with the parent proteins. Reactivity: +++, strong; ++, fair; +, weak; −, negative.

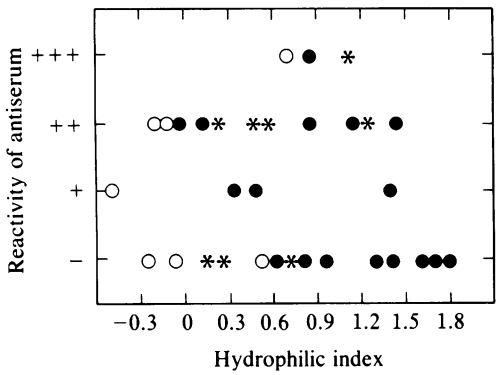

FIG. 4. Effect of peptide hydrophilicity on the reactivities of antisera to oncoproteins. Location of peptide in parental oncoprotein: ○, NH_2-terminal; ●, CO OH-terminal; *, middle. Reactivity: +++, strong; ++, fair; +, weak; −, negative.

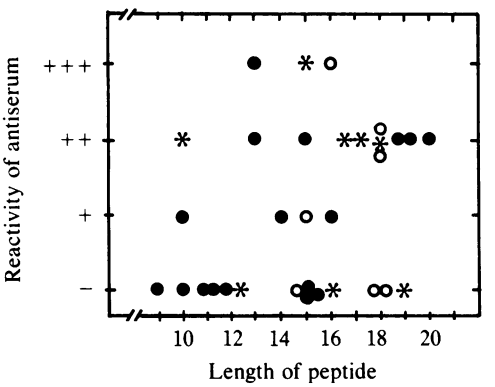

FIG. 5. Effect of length of the immunizing peptides (number of amino acids) on the reactivities of antisera to oncoproteins. Location of peptide in the parental oncoprotein: ○, NH_2-terminal; *, middle; ●, COOH-terminal regions. Reactivity: +++, strong; ++, fair; +, weak; −, negative.

for the production of useful antibodies. What factors determine the production of protein-reactive antipeptide antibodies? The length of the peptides, their hydrophilicity, their location in the native molecule, the secondary and tertiary configuration of the peptides may all be important factors in generating useful antibodies.

In this study we chose 35 peptides from several oncoproteins to examine their immunogenicity and ability to induce antibodies reactive with the corresponding regions on protein molecules. The peptides were selected on the basis of their hydrophilicities in the range of −0.52 to +1.80. It is noteworthy that no differences among hydrophilic indices of peptides in this range appeared to be important for immunogenicity.

After coupling to keyhole limpet hemocyanin as a carrier, 32 of 35 peptides (91%) induced antipeptide antibodies in rabbits. It should be noted that all peptides with fewer than 10 amino acid residues failed to induce effective antibody responses. On the other hand, (97%) of the peptides with more than 10 amino acid residues induced antibody responses of high titer (Fig. 1). This result is consistent with other observations that size is an important factor in the effectiveness of peptides as immunogens (1, 23). Thus it is important that only 1 of 7 peptides made up of fewer than 13 amino acids produced antibodies that reacted strongly with native proteins. This point has not been stressed frequently in the literature in spite of the fact that several investigators have observed a similar phenomenon. Van Eldik *et al.* (24) generated antibodies that reacted with calmodulin by utilizing a decapentapeptide but failed to do so with a heptapeptide. Two octapeptides failed to generate antibodies against influenza hemagglutinin, whereas two peptides with 18 or 27 amino acids were effective (25). The observations of Niman *et al.* (7) are also pertinent. These investigators made monoclonal antibodies to six different peptides. Antibodies to two peptides comprised of 30 and 36 amino acids reacted with native protein in 2 out of 2 and 16 out of 21 cases, respectively. On the other hand, with antibodies to four peptides comprised of from 13 to 16 amino acids, reactivity with protein varied between 1 out of 4 cases to 3 out of 6 cases. These observations suggest that the likelihood of generating useful heteroantibodies that react with native protein increases with the size of the peptide.

In the current study, more than one-half of the antipeptide antisera reacted with their respective oncoproteins derived from virus-transformed cell lines. However, among four anti-myc peptide antisera with high titers to peptides ($1:10^4$), only one reacted with the $p110^{gag\text{-}myc}$ oncoprotein while four out of five anti-*fes*-peptide antisera reacted with the $p85^{gag\text{-}fes}$ oncoprotein. The difference among the reactivities of different antipeptide antisera with native protein presumably depends on whether or not the region selected for synthesis of the peptide corresponds to an antigenic domain on the native protein molecule. Although regional hydrophilicity helps in selecting the peptide, there is as yet no way to predict a useful antigenic domain with certainty. Altman *et al.* (26) reported that only four out of seven (57%) antisera, raised in rabbits to interleukin 2 peptides of 13 to 15 amino acids in

Table 2. Effects of mimicking sequence of peptides on specificities of antibodies generated

Peptide	Oncogene	Amino acid sequence	Position	Cross-reactivity between antisera and peptides	Reactivity to oncoprotein
4	v-Ki-*ras*	M-T-E-Y-K-L-V-V-V-G-A-S-G-V-G-K-S-A	1–18	Each antiserum cross-reacted with the other three peptides	++ (v-Ki-*ras* and v-Ha-*ras*)
8	v-Ha-*ras*	M-T-E-Y-K-L-V-V-V-G-A-R-G-V-G-K-S-A			−
9	c-Ha(EJ)-*ras*	M-T-E-Y-K-L-V-V-V-G-A-V-G-V-G-K-S-A			−
10	c-Ha-*ras*	M-T-E-Y-K-L-V-V-V-G-A-G-G-V-G-K-S-A			++ (v-Ha-*ras*)
33	v-*myc*	R-L-I-A-E-K-E-Q-L-R-R-R-R-E-Q	389–403	No cross-reactivity	++
34		E-Q-L-R-R-R-R-E-Q-L-K	395–405		−
27	v-*fes*	A-S-P-Y-P-N-L-S-N-Q-Q-T-R	893–905	No cross-reactivity	++
28		N-Q-Q-T-R-E-F-V-E-K-G-G-R	901–913		+++
19	v-*myb*	P-S-P-P-V-D-H-G-C-L-P-E-E-S-A-S-P-A-R	168–186	Cross-reacted	−
20		P-P-V-D-H-G-C-L-P-E-E-S-A-S-P-A	170–185		−
21	v-*myb*	P-F-H-K-D-Q-T-F-T-E-Y-R-K-M	247–260	Cross-reacted	+
22		P-F-H-K-D-Q-T-F-T-E-Y-R-K-M-H-G-G-A-V	247–265		++

length, reacted with native protein. This rate of generating protein-reactive antipeptide antibodies was compatible with our observations.

There is an assumption that the COOH-terminal region of many proteins is relatively free to rotate and might be more exposed and free to react with antibodies than other regions of the molecules. To test this assumption we compared the reactivity of antipeptide antisera according to peptide locations in native molecules (Fig. 3). It was clear that the ability of antipeptide antisera to react with proteins was independent of peptide location in the molecule.

Several other observations were of interest. Despite apparently adequate length (16–19 amino acids) and hydrophilic indices (+0.22 and +0.23) some peptides such as myb peptides 19 and 20 did not induce antibodies that could react either with the native protein, $p48^{myb}$, as tested by immunoprecipitation or with extensively denatured proteins as tested by immunoblotting. Other investigators have also reported failure of antipeptide antisera to react with the corresponding protein despite the apparent adequate size and hydrophilicity of the peptides (26, 27).

In the case of v-*myc* peptides 33 and 34, corresponding to residues 389–403 and 395–405, and v-fes peptides 27 and 28, corresponding to residues 893–905 and 901–913, the antisera reacted with the homologous but not the heterologous peptide despite sharing a nine or a five amino acid sequence, respectively. An antigenic region of myc oncoprotein is probably associated with part of the region of residues 389–403 because antiserum to peptide 33 containing these residues reacted with $p110^{gag\text{-}myc}$, whereas, antibody to peptide 34 (residues 395–405) was unreactive with this oncoprotein. The antisera to fes peptides 27 and 28, although not reactive with heterologous peptides, did react with the v-fes protein, $p85^{gag\text{-}fes\text{-}GT}$, suggesting that the two antisera recognized different domains of the v-fes protein.

Of particular interest are the observations relating to the first 18 amino acids of various ras peptides that differ in a single amino acid at position 12. As anticipated, antiserum to each peptide cross-reacted with all other peptides. A surprising observation was that antiserum to peptides 8 and 9 failed to react with any ras oncoproteins despite the similarity of their sequences to those of peptides 4 and 10. Peptide 4 generated antibodies reactive with p21 in both NRK-K and NRK-H while antiserum to peptide 10 reacted with p21 only in NRK-H. It seems likely that the predominant species of reactive antibody molecules in these four antisera were generated by different immunogenic domains of four peptides, each differing in a single amino acid.

It was noteworthy that the substitution of valine in c-Ha(EJ)-*ras* for the glycine in normal c-Ha-*ras* resulted in the production of antisera of considerably divergent reactivities. The conformational change is remarkable when this single amino acid substitution occurs at this position in the naturally occurring p21 molecule (28). It is possible that some antibodies might not bind to the protein molecule as a consequence of this change in conformation. Our observations concerning the four peptides that differ in a single amino acid among 18 should be compared with those of Alexander *et al.* (27). These investigators used two octadecapeptides differing in a single amino acid to immunize rabbits. Antisera to each of the peptides reacted more strongly with the homologous peptide than with the heterologous peptide and, in addition, one of the antisera was capable of recognizing the corresponding single amino difference in the native protein.

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