

# Amino acid residues essential for biological activity of a peptide derived from a major histocompatibility complex class I antigen

(glucose transport/ordered structure)

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**ABSTRACT** The stimulatory activity of peptides from the  $\alpha 1$  domain of the major histocompatibility complex (MHC) class I antigen on adipose cell glucose transport was previously shown to require a preformed, ordered conformation of the peptide. The two peptides studied previously were D<sup>k</sup>-(61–85) (ERETQIAKGNESFRVLDLRTLLRY) and D<sup>k</sup>-(69–85). We now show that systematic alanine substitution in D<sup>k</sup>-(69–85) identifies residues that are essential for biological activity. Ordered structure of the peptides, estimated by circular dichroism, was found in all peptides with activity, but with a complex variety of spectra. Inactive peptides were in either a random coil or an ordered structure. Ordered structure, therefore, is not sufficient for activity. The peptides self-interact in the absence of cells and form aggregates that precipitate upon centrifugation. The tendency to aggregate is correlated with biological potency. Only MHC class I molecules have significant homology to the peptides studied here. The peptide self-interaction suggests that the biological effects in cells, which result from inhibition of receptor and transporter internalization, may be due to the binding (tantamount to self-interaction) of the peptide to the homologous sequences in the  $\alpha 1$  domain of the MHC class I molecule.

Previous studies demonstrated that certain peptides derived from the  $\alpha 1$  domain of major histocompatibility complex (MHC) class I antigens [e.g., D<sup>k</sup>-(61–85)] enhance insulin-stimulated glucose transport in adipose cells. This effect is due to an increased number of active insulin receptors (1) and glucose transporters (13) on cell surfaces consequent to inhibition of receptor and transporter internalization (1–3). D<sup>k</sup>-(61–85) is active only if it has assumed an ordered conformation prior to its interaction with cells (2). The initial findings were with 25-residue peptides, but it was found subsequently that the eight N-terminal residues could be removed without loss of activity (1). We now identify residues in D<sup>k</sup>-(69–85) that are essential for activity and others that are not. In addition, we show that while inactive peptides may adopt either ordered structure or random coil conformation, active peptides must have an ordered structure. Peptide molecules in ordered structure interact with themselves to a greater or lesser extent, forming aggregates that precipitate upon centrifugation. Biological activity of the peptides is correlated with the tendency to form aggregates. We discuss the significance of the observations in light of the fact that the peptides have sequence similarity only to MHC class I molecules (4, 5) among known protein sequences.

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## MATERIALS AND METHODS

**Glucose Transport in Adipose Cells.** The biological activity of the peptides was measured by their effect on glucose uptake in rat adipose cells as described elsewhere (1). Briefly, rat adipose cells were obtained from epididymal fat pads and suspended in Krebs–Ringer Hepes buffer with 5% bovine serum albumin at a lipocrit of 10% (final). The peptide effect was measured in cells maximally stimulated with insulin (8 nM). After equilibration at 37°C for 30 min the cells were incubated for 30 min at 37°C with buffer (basal), 8 nM insulin, or 8 nM insulin plus peptide. D-[<sup>14</sup>C]glucose was added, and the cells were incubated for an additional 30 min and harvested on oil. Biological activity was measured by a dose-response curve to interpolate the EC<sub>50</sub> value in the usual way, taking the maximum enhancement of insulin effect (about 40% over the insulin-only maximum) as 100%. Most of the peptides were not tested at concentrations higher than 30  $\mu$ M. Peptides that enhanced the maximum insulin effect by less than 20% at 30  $\mu$ M were considered inactive. Accordingly, three categories of peptides were defined: those with full activity, EC<sub>50</sub> < 10  $\mu$ M; reduced activity, 10  $\mu$ M  $\leq$  EC<sub>50</sub>  $\leq$  30  $\mu$ M; no activity, EC<sub>50</sub> > 30  $\mu$ M.

**Peptides.** The peptides were assembled stepwise either on a phenylacetamidomethyl (PAM) resin using the *t*-Boc NMP/HOBt protocol of an Applied Biosystems model 430A peptide synthesizer or on a *p*-alkoxybenzyl alcohol (Wang) resin using a modified Fmoc/BOP protocol of a Milligen/Biosearch Model 9050 synthesizer. The side-chain-protecting groups were as follows: *t*-Boc chemistry, *N*<sup>ε</sup>-mesitylene-2-sulfonyl for Arg,  $\beta$ -cyclohexyl ester for Asp,  $\gamma$ -benzyl ester for Glu, *N*<sup>ε</sup>-2-chlorobenzoyloxycarbonyl for Lys, *O*-benzyl for Ser and Thr; and *O*-2-bromobenzoyloxycarbonyl for Tyr; Fmoc chemistry, 4-methoxy-2,3,6-trimethylbenzenesulfonyl for Arg,  $\beta$ -*t*-butyl ester for Asp,  $\gamma$ -*t*-butyl ester for Glu, *N*<sup>ε</sup>-*t*-butoxycarbonyl for Lys, and *O*-*t*-butyl for Ser, Thr, and Tyr. The *t*-Boc-assembled peptides were deprotected/cleaved from the solid support by using HF in the presence of anisole, ethanedithiol, and dimethyl sulfide as scavengers. After conversion of the hydrofluoride to the acetate salt by ion-exchange column chromatography, the peptides were purified to greater than 98% homogeneity by preparative high-performance liquid chromatography using a Vydac C<sub>18</sub> (2.2  $\times$  25 cm) column and appropriate linear gradients of 0.1% trifluoroacetic acid (TFA)-buffered acetonitrile in 0.1% aqueous TFA. The Fmoc-assembled peptides were deprotected/cleaved from the resin by using TFA in the presence of thioanisole, ethanedithiol, water, and phenol as scavengers and were purified by preparative high-performance liquid chromatography as described above. The desired peptides

Abbreviation: MHC, major histocompatibility complex.  
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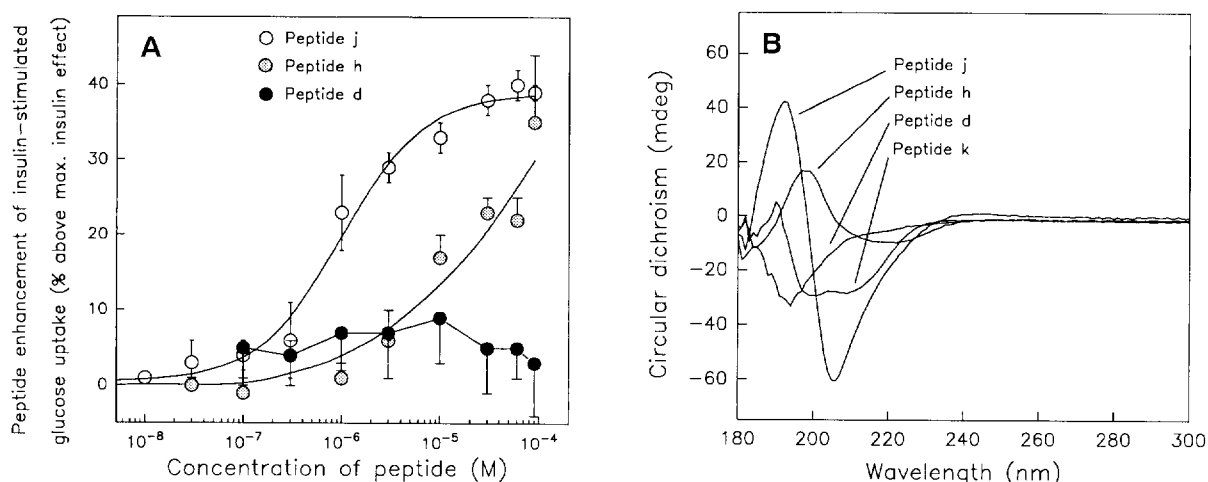


FIG. 1. (A) Representative dose-response curves for peptides with full biological activity (○), reduced activity (◐), or no activity (●). The values are mean  $\pm$  SEM of three experiments with triplicate samples at each point. (B) CD spectra for 1 mM solutions of the three peptides in A and of peptide k. The measurements for each peptide were done twice at room temperature. The results of one set of experiments are shown.

were confirmed by sequence analysis, amino acid composition, and fast atom bombardment mass spectrometry. We reported previously that the MHC-derived peptides may occur in both an active and an inactive conformation. Accordingly, the peptides used in the present study were activated by incubation of 1 mM stock solution at 37°C in 0.1 M NaCl overnight, as described (2).

**Circular Dichroism (CD).** CD spectra were recorded on a Jasco (Easton, MD) J-600 calibrated against *d*-camphorsulfonic acid using  $\Delta\epsilon$  (290.5 nm) = +2.38 M<sup>-1</sup>·cm<sup>-1</sup>. Rectangular cuvettes with path lengths of 0.01 cm were used for recording spectra of 1 mM peptide stock solutions.

**Aggregation.** Peptide stock solution was diluted in Krebs-Ringer Hepes pH 7.2 buffer to 30  $\mu$ M, incubated (30 min, 37°C), then centrifuged at 12,000  $\times$  *g* for 10 min. The amount of peptide remaining in solution was measured spectrophotometrically by absorbance at 278 nm ( $\epsilon$  = 1200 M<sup>-1</sup>·cm<sup>-1</sup> per tyrosine residue).

## RESULTS

**Biological Activity.** We analyzed D<sup>k</sup>-(62-85) and D<sup>k</sup>-(69-85) by systematic replacement of residues with alanine (alanine scan) (6-8) to assess the importance of each residue for

Table 1. Sequence, biological activity, and aggregation of MHC class I-derived peptides

Peptide	Code	Sequence	Residue(s) replaced	EC <sub>50</sub> ,* μM	Aggregation,† %
D <sup>k</sup> -(62-85)	a	RETQIAKGNEQSF <sup>62</sup> RV <sup>65</sup> DLRTLLRY <sup>70</sup>		2	70 $\pm$ 2
D <sup>k</sup> -(69-85)	b	GNEQSF <sup>69</sup> RV <sup>75</sup> DLRTLLRY <sup>80</sup>		5	NT
Single substitutions					
[Ala <sup>71</sup> ]D <sup>k</sup> -(69-85)	c	GNA <sup>71</sup> QSF <sup>75</sup> RV <sup>80</sup> DLRTLLRY <sup>85</sup>	E	1	65 $\pm$ 6
[Ala <sup>74</sup> ]D <sup>k</sup> -(62-85)	d	RETQIAKGNEQSA <sup>74</sup> RV <sup>78</sup> DLRTLLRY <sup>83</sup>	F	>30	33 $\pm$ 4
[Ala <sup>78</sup> ]D <sup>k</sup> -(69-85)	e	GNEQSF <sup>78</sup> RV <sup>82</sup> DLRTLLRY <sup>87</sup>	L	30	39 $\pm$ 6
[Ala <sup>81</sup> ]D <sup>k</sup> -(69-85)	f	GNEQSF <sup>81</sup> RV <sup>85</sup> DLRTLLRY <sup>90</sup>	L	>30	64 $\pm$ 6
[Ala <sup>82</sup> ]D <sup>k</sup> -(69-85)	g	GNEQSF <sup>82</sup> RV <sup>86</sup> DLRTLLRY <sup>91</sup>	L	>30	35 $\pm$ 4
[Ala <sup>83</sup> ]D <sup>k</sup> -(69-85)	h	GNEQSF <sup>83</sup> RV <sup>87</sup> DLRTLLRY <sup>92</sup>	R	30	91 $\pm$ 1
[Ala <sup>84</sup> ]D <sup>k</sup> -(69-85)	i	GNEQSF <sup>84</sup> RV <sup>88</sup> DLRTLLRY <sup>93</sup>	Y	10	89 $\pm$ 2
[Ala <sup>85</sup> ]D <sup>k</sup> -(69-85)	j	GNEQSF <sup>85</sup> RV <sup>89</sup> DLRTLLRY <sup>94</sup>	Y	1	88 $\pm$ 5
Double substitutions					
[Ala <sup>68,75</sup> ]D <sup>k</sup> -(62-85)	k	RETQIA <sup>68</sup> AGNEQSF <sup>75</sup> AV <sup>79</sup> DLRTLLRY <sup>84</sup>	K, R	>30	44 $\pm$ 6
[Ala <sup>69,76</sup> ]D <sup>k</sup> -(62-85)	l	RETQIAK <sup>69</sup> ANEQSF <sup>76</sup> RA <sup>80</sup> DLRTLLRY <sup>85</sup>	G, V	30	42 $\pm$ 4
[Ala <sup>70,77</sup> ]D <sup>k</sup> -(62-85)	m	RETQIAK <sup>70</sup> AG <sup>77</sup> EQSF <sup>81</sup> RV <sup>85</sup> AL <sup>84</sup> RTLLRY <sup>89</sup>	N, D	7	72 $\pm$ 4
[Ala <sup>72,79</sup> ]D <sup>k</sup> -(62-85)	n	RETQIAKGNE <sup>72</sup> AS <sup>79</sup> RV <sup>83</sup> DL <sup>87</sup> ATLLRY <sup>91</sup>	Q, R	>30	54 $\pm$ 4
[Ala <sup>73,80</sup> ]D <sup>k</sup> -(62-85)	o	RETQIAKGNEQ <sup>73</sup> AF <sup>80</sup> RV <sup>84</sup> DL <sup>88</sup> RA <sup>92</sup> LLRY <sup>96</sup>	S, T	>30	59 $\pm$ 4
Other peptides					
HLA-A2-(69-85)	p	AHSQTHR <sup>69</sup> V <sup>73</sup> DLG <sup>77</sup> TLRG <sup>81</sup> YY <sup>85</sup>		>30	NT
HLA-A2-(69-76)-D <sup>k</sup> -(77-85)	q	AHSQTHR <sup>69</sup> V <sup>73</sup> DLRT <sup>77</sup> LLRY <sup>81</sup>		>30	NT
D <sup>k</sup> -(69-76)-HLA-A2-(77-85)	r	GNEQSF <sup>69</sup> RV <sup>73</sup> DLG <sup>77</sup> TLRG <sup>81</sup> YY <sup>85</sup>		10	NT
HLA-B27-(69-85)	s	AKAQT <sup>69</sup> D <sup>73</sup> RED <sup>77</sup> LR <sup>81</sup> LLRY <sup>85</sup>		>30	NT
[Phe <sup>74</sup> ]HLA-B27-(69-85)	t	AKAQT <sup>69</sup> F <sup>74</sup> RED <sup>78</sup> LR <sup>82</sup> LLRY <sup>86</sup>		1	NT

\*EC<sub>50</sub> value for glucose uptake as measured in the rat adipose cell assay. Maximal peptide effect in cells fully stimulated by insulin was 40% enhancement over insulin alone. Peptides giving less than 20% enhancement at 30  $\mu$ M were considered inactive (EC<sub>50</sub> > 30  $\mu$ M).

†Aggregation was measured by centrifugation of 30  $\mu$ M peptide solution in Krebs-Ringer Hepes for 10 min at 12,000  $\times$  *g* and the amount of peptide remaining in solution was determined spectrophotometrically. The numbers indicated are percent precipitated and are mean  $\pm$  SEM of three experiments. NT, not tested.

biological activity. Fig. 1A shows three typical dose-response curves for stimulation of glucose uptake, for a fully active peptide (j), a peptide with reduced activity (h), and an inactive peptide (d). (See Table 1 for alphabetical coding.) Table 1 presents the potency of D<sup>k</sup>-(62–85) and D<sup>k</sup>-(69–85), of 13 peptides in the alanine scan, and of 5 additional peptides of interest. Substitution for Phe<sup>74</sup> (d), Leu<sup>81</sup> (f), and Leu<sup>82</sup> (g) each resulted in loss of activity. Peptide d was completely inactive even at 90 μM, the highest concentration that was tested. Peptides with alanine instead of Leu<sup>78</sup> (e), Arg<sup>83</sup> (h), or Tyr<sup>84</sup> (i) all had reduced activity compared with D<sup>k</sup>-(69–85) (b). Replacement of Glu<sup>71</sup> (c) or Tyr<sup>85</sup> (j) yielded peptides that were even more potent than the original; this is an interesting result in particular for peptide j, as we had shown previously that C-terminal truncation by deleting Tyr<sup>85</sup> results in considerable loss of activity (2).

The alanine scan with double residue changes showed (in m) that neither Asn<sup>70</sup> nor Asp<sup>77</sup> is important for activity. Peptide l had reduced activity, but the data do not allow a conclusion as to whether Gly<sup>69</sup> or Val<sup>76</sup> is most important. The three other peptides with double alanine substitutions (k, n, o) were all inactive. In peptide k the essential residue is likely to have been Arg<sup>75</sup>, as residues 62–68 can be deleted entirely (cf. a and b) without loss of activity. The data did not allow a decision as to whether the inactivity of peptides n and o was due to substitution of only one or both of the residues. However, as the chimeric peptide r, with Gly<sup>79</sup>, was moderately active, Gln<sup>72</sup> seems more responsible than Arg<sup>79</sup> for the loss of activity in peptide n.

The essential role of Phe<sup>74</sup> is shown not only by the inactivity of d, and of both p and q (which contain many of the other residues shown to be essential), but most dramatically by the fact that the inactive human peptide s became fully active when Asp<sup>74</sup> was changed to Phe<sup>74</sup> (peptide t).

**CD.** We showed previously (2) that D<sup>k</sup>-(61–85) is active only if it has assumed an ordered conformation prior to interaction with cells. Therefore residues identified in the alanine scan as important for biological activity might be essential for maintaining an ordered structure, for interaction with a binding site, or for both. Measurements at 1 mM in 0.1 M NaCl yielded a variety of complex CD spectra, so that simple classification into recognized structures [such as α-helix (9, 10)] was often not possible.

Fig. 1B shows typical CD spectra of four peptides (three of them the same as in Fig. 1A), selected to show the variable features. Peptide j, which is fully active, has a spectrum with maxima at 205 nm (negative) and 195 nm (positive), suggesting a high content of ordered structure. The original unsubstituted peptides a and b (see ref. 2) as well as the two other most active peptides (c and t) have a similar positive CD signal at 195 nm.

Peptides h and k, with reduced activity and no activity, respectively, have CD spectra with both a positive and negative maximum, but without the typical profile of peptide j. Peptides e, i, and r, all of which have reduced activity, and the inactive peptides (f, n, o, and s) also fall into this category. The CD spectrum of peptide m is also in this category, although its activity is comparable to that of peptide b. Peptides g and q (no activity) and peptide l (reduced activity) have spectra with a negative maximum, no positive maximum, but with an indication of some molecules with ordered structure.

Only the inactive peptides d and p have the typical spectrum of a random coil, with a negative maximum at 195 nm.

Analysis of the data in Fig. 2A shows a correlation between the degree of ordered structure as estimated by CD and the biological activity ( $\chi^2 = 10.6$ ;  $P < 0.05$ ), by a conservative test ignoring the rank order of categories in the 3 × 3 contingency table.

**Aggregation.** Table 1 shows the extent of aggregation of the various peptides. The scatter diagram in Fig. 2B indicates a positive correlation ( $r = 0.56$ ;  $P < 0.05$ ) of biological activity with the ability of the peptides to self-interact and form aggregates. Fig. 2C shows that peptide self-interaction (aggregation) and the degree of ordered structure also are correlated ( $r = 0.49$ ;  $P < 0.05$ ).

## DISCUSSION

The present results, summarized in Fig. 3A, are consistent with and extend our earlier observations (2) on the gain and loss of activity associated with a reversible conformation change of D<sup>k</sup>-(61–85), which is active only if it assumes an ordered structure prior to its interaction with cells. In the present series, ordered structure was correlated with potency, and all peptides with activity had ordered structure.

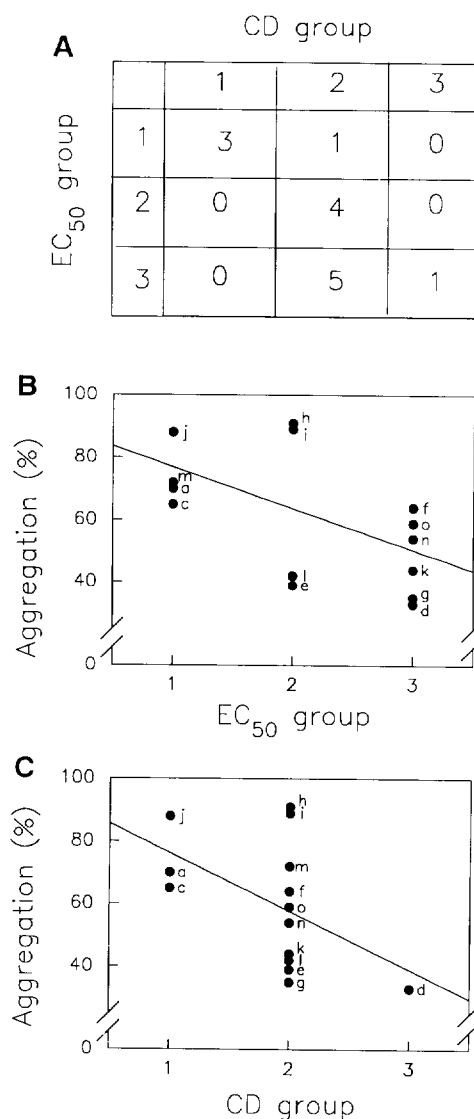


FIG. 2. Correlation between biological activity, ordered structure, and aggregation. (A) Biological activity and CD signal. Peptides were categorized into three groups according to EC<sub>50</sub> values (see also *Materials and Methods*): 1, full activity; 2, reduced activity; 3, no activity. For CD spectra, three groups were defined: 1, spectrum comparable to that of peptide j (see Fig. 1B); 2, intermediate spectrum; 3, random coil. (B) Biological activity and aggregation. (C) CD spectra and aggregation. The three groups for biological activity and CD spectra are the same as in A. Aggregation values are from Table 1. Regression lines are shown.



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