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Discovery of D-amino-acid-containing ligands with selectide technology

(Synthetic peptide library; antibody; streptavidin; mimotope)

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SUMMARY

When all L-amino acid (aa) random peptide libraries synthesized on solid-phase particles were screened (Selectide Technology), we identified several peptide ligands (YG_F_) that interacted specifically with an anti-β-endorphin monoclonal antibody (clone 3E7) (single-letter aa symbols; symbols '_' indicate variable aa). Here, we report on the screening of three different D-aa-containing pentapeptide libraries (XxXxX, xXxXx and xxxxx, wherein X = L-aa, and x = D-aa) with the same antibody, in which several D-aa-containing ligands were identified. The binding affinities of many of these D-aa-containing ligands were as least two orders of magnitude lower than that of YGGFL, for which the K_i is 17.5 nM.

INTRODUCTION

Over the last few years, enormous effort has been given to the development of new methods to rapidly discover peptide ligands for target macromolecules. These methods center around the idea of library screening, that is, to select specific peptide ligands from a large collection of random or semi-random peptide ligands. In general, there are two methods of constructing peptide libraries: (*i*) filamentous phage approach using molecular biology techniques (Cwirla et al., 1990; Devlin et al., 1990; Scott et al., 1990), and (*ii*) synthetic peptide approach using solid phase peptide synthesis (Geysen et al., 1986; 1987; Fodor et al., 1991; Furka et al., 1991; Houghten et al., 1991; 1992; Lam et al., 1991; 1992; 1993; Lam and Lebl, 1992). Both methods have successfully proven to be useful in discovering peptide ligands specific for various target macromolecules: streptavidin (Devlin et al., 1990; Lam et al., 1991; Lam and Lebl, 1992), avidin (Lam and Lebl, 1992), mAb (Geysen et al., 1986; 1987; Cwirla et al., 1990; Scott et al., 1990; Houghten et al., 1991; 1992; Lam et al., 1991; 1992; 1993), concanavalin A (Oldenberg et al., 1992; Scott et al., 1992), opiate receptor (Houghten et al., 1992), and peptides with antibacterial activities (Houghten et al., 1991). The major limitation of the filamentous phage technique, however, is that the library has to consist of only genetically encoded natural aa since a biological system is used, whereas in the synthetic peptide library approach, unnatural aa can easily be incorporated into the constructs of the library. We described the use of a 'split-synthesis' approach to synthesize a random peptide library such that each bead expresses a single peptide entity. We then successfully demonstrated that using an enzyme-linked immunoassay technique, we were able to

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Abbreviations: a, D-alanine; A, L-alanine; aa, amino acid(s); β, β-alanine; βEN, β-endorphin; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexa-fluorophosphate; DIEA, diisopropylethylamine; DMF, dimethylformamide; ε, ε-aminocaproic acid; EN, endorphin; f, D-phenylalanine; F, L-phenylalanine; Fmoc-aa, 9-fluorenyl-methoxycarbonyl amino acids; G, glycine; HOBt, *N*-hydroxybenzotriazole; K_i, inhibition binding constant; mAb, monoclonal antibody(ies); q, D-glutamine; Q, L-glutamine; t, D-threonine; w, D-tryptophan; W, L-tryptophan; x, all 18 D-aa plus glycine without cysteine; X, all 18 L-aa plus glycine without cysteine; y, D-tyrosine; Y, L-tyrosine; _, variable aa.

isolate specific ligand for various macromolecular targets (Lam et al., 1991; 1992; 1993; Lam and Lebl, 1992). In this paper, we report on the use of this technique (Selectide Technology) to generate D-aa-containing pentapeptide libraries, from which several D-aa-containing mimotopes were discovered for an anti-β-endorphin (anti-βEN) mAb (clone 3-E7).

RESULTS AND DISCUSSION

(a) Anti-β-endorphin mAb and its known binding motif

The target macromolecule used in our peptide library screening was an anti-βEN murine mAb (clone 3-E7). The hybridoma that secretes this mAb was developed by Meo et al. (1983) and the immunogen used was synthetic human βEN. This mAb recognizes the tetrapeptide YGGF that represents the message sequence located at the N-terminus of all naturally occurring mammalian opioid peptides (Meo et al., 1983).

We have previously reported (Lam et al., 1991; 1992; 1993) the use of this mAb in the screening of synthetic all L-aa peptide libraries (4-mer to 9-mer) and the general motif of YG(G/A)(F/L/M/W) was elucidated. However, for higher affinity ligands, the motif appeared to be more restrictive: YG(G/A)F. Some of the higher-affinity ligands isolated have binding affinities similar to that of YGGFL (the native epitope) for which the $K_i = 17.5$ nM (Lam et al., 1991). Cwirla et al. (1990) also reported the use of this same mAb in the screening of a hexapeptide library but using a filamentous phage approach instead. In their study, 51 ligands were isolated with a general motif of YG_ _ _ _. Only nine of the 51 ligands had an L-Phe at the fourth position, and the binding affinity of the best ligand isolated was 50-times lower than that of YGGFL. However, more recently, using a low concentration of 3E7 Fab fragment, Barrett et al. (1992) were able to isolate high affinity YGGFL and closely related ligands. Fodor et al. (1991) used a light-directed, spatially addressable parallel chemical synthesis method to synthesize 1024 analogs of YGGFL and use an immunofluorescent method with the same anti-βEN mAb to analyze the binding, and the following preferred binding sequence for this antibody was deduced: YG(A/G)(F/L).

(b) D-aa-containing ligands

The D-aa-containing penta-peptide libraries used in our screening for anti-βEN were: (*i*) XxXxXβεβε-resin, (*ii*) xXxXxβεβε-resin, and (*iii*) xxxxxβεβε-resin. In these libraries, there should be $19^5 = 2\,476\,099$ possible permutations each. However, only approximately 500 000 beads were screened in each of these libraries and therefore not all possible permutations were included. Nonetheless, several ligands were isolated and sequenced and the results are summarized in Table I.

Under identical screening conditions, with these three D-aa-containing peptide libraries, the XxXxX library yielded the most ligands. As indicated earlier, the binding motif for this anti-βEN mAb is YG_F, and since Gly is achiral, the probability of YGXxX in this XxXxX-library is $1/19^2 = 1/361$. From the L-aa library screen, L-Phe appears to be critical in the fourth position. However, this is not possible in this library as the fourth position can only be a D-aa. It is intriguing to find that for seven of the 10 isolated ligands, with an N-terminal L-Tyr, L-Phe was found in either the third or fifth position. In one case, YGFGF, L-Phe is present in both the third and the fifth position with Gly sandwiched in between. In three other cases, YGGfM, YGAfW and YGAfF, this mAb appears to be able to accommodate D-Phe in the fourth position instead. However, there is another possible explanation for the presence of D-Phe at position 4. For instance, when we screened an L-pentapeptide library with anti-βEN, a motif with L-Tyr at position 1 and L-Phe at position 5 was discovered: YGEAF, YGGGF, YGHAF, YGLGF, YGMGF, YKGGF, YLGGF and YQGGF (Lam et al., 1993). In all these cases, the increase distance between Tyr and Phe is 'compensated' by the presence of conformational flexible aa (two Gly or at least Gly and Ala) in the sequence connecting the two aromatic residues. We have defined earlier the binding motif as YG(G/A)(F/L/M/W) where position 4 could be Phe, Leu, Met, or Trp (Lam et al., 1993). It is therefore conceivable that D-Phe (in the case of YGGfM, YGAfW, and YGAfF) and D-Ala (in the case of YGAaF) are the only aa tolerable at position 4 and the true contact residue of these ligands may indeed be the fifth residue (Met, Trp, or Phe).

One ligand, IyGGF, is interesting as the L-Tyr is no longer in the N-terminal position, but instead it is the second residue and it is a D-isomer. So far, all nine of these ligands are somehow related to the motif YG_F. However, the remaining two ligands, YaNaW and YaQaW, although still with an N-terminal L-Tyr, are structurally quite unique and they fall into a new motif of Ya(N/Q)aW. This is particularly important as it is very unlikely that this motif can be discovered by any other means, either by the filamentous phage peptide library approach, or by conventional serial structure-activity-relationship analysis approach. It is interesting that Asp and Glu are interchangeable suggesting that the amide side chain is critical at position 3 for this motif. It is conceivable that the two Ya(N/Q)aN peptides interact with a contact residue(s), different from that of YG_F on the antibody paratope. However, topologically those contact residues are probably not too far away from those interacting with the L-Phe of YG_F as the N-terminal L-

TABLE I

D-aa-containing ligands that interact specifically with anti-βEN mAb (clone 3-E7)

Library[a]	Ligands isolated
XXXXX-linker-resin	YG_F_ YG(G/A)(F/L/M/W)_
XxXxX-linker-resin	YGGfM YqGGF YGFGL IyGGF YaNaW
	YGAfW YGAaF YGFGF YaQaW
	YGAfF YGGGF
xXxXx-linker-resin	yGGFv, yGGFa
xxxxx-linker-resin	wtGGy

[a]The above random peptide libraries were synthesized with a split synthesis method as described before (Lam et al., 1991; 1992). In brief, after the linker was synthesized on the resins, the resins were aliquoted equally into 19 different reaction vials to which fourfold excesses of Fmoc-aa were added, followed by BOP, HOBt and DIEA. After coupling was complete, the resins were mixed, washed, and deprotected with piperidine. After thorough washing, the resins were again distributed and Fmoc-aa coupled as described. The randomization steps were repeated for three additional cycles with the appropriate D- or L-aa, for a total of five coupling cycles. The side chain protecting groups were finally removed with a mixture of trifluoroacetic acid, phenol, anisole, and ethanedithiol (94:2:2:2; v/w/v/v). The resin was then washed with DMF and neutralized with 10% DIEA in DMF. After thorough washing with double distilled water, the resins were ready to be screened with anti-βEN as described before (Lam et al., 1991). The positive color beads were physically isolated and microsequenced with a protein sequencer (Model 477A, Applied Biosystems).

Tyr were conserved in both cases, and both of these motifs compete with YGGFL for binding.

When an xXxXx penta-library containing 500 000 beads was screened, only two ligands were isolated: yGGFv and yGGFa. This motif is related to that of YG_F_ except that the N-terminal is now D-Tyr instead of the L-isomer. Again the Tyr and Phe are separated by two aa.

When an all D-aa library containing 500 000 beads was screened, only one ligand was isolated, wtGGy. This ligand is particularly intriguing as it is _ _ GGy instead of YGG_ _, a reverse in polarity for a D-isomer. Interestingly, although in this all D-aa pentapeptide library, where yG_f is present with a probability of $1/19^3 = 1/6859$, none of the peptides with this motif are discovered suggesting that the antibody can accommodate YG_F, YG_f, or yG_F (see above), but not yG_f, with both Tyr and Phe in the D-isomeric form.

(c) Binding affinities

Since the effective concentration of peptide on the surface of the solid-phase bead is believed to be very high (Lam et al., 1993), and a bivalent immunoglobulin was used in the screen, ligands with very low binding affinities can still be detected although usually with a lighter stain. Therefore, it is not surprising that the binding affinities of many of these D-aa ligands are quite low. In fact, the peptide ligands that have any significant measurable binding affinities by univalent competitive radioimmunoassay (Lam et al., 1991), are YGAfF and YGAaF (K_i is estimated to be 1 μM and 10 μM, respectively, which is two to three orders of magnitude weaker than that of YGGFL, for which the K_i is 17.5 nM).

(d) Advantages and limitations of synthetic peptide libraries

As indicated earlier, the major limitation of the filamentous phage peptide library approach is that only L-aa libraries can be used in the screen. The synthetic peptide library approach, on the other hand, not only can include unnatural aa, such as D-aa, *N*-methylated aa, glycoamino acids, and *N*-substituted Gly, but can also incorporate various secondary structures, such as cyclics (via disulfide, β-lactam, or alkylation; Inman et al., 1991), α-helices, or β-sheets into the constructs of the peptide libraries. The major limitation of the synthetic library approach, however, is that it would be impractical to synthesize a library of greater than 20-mers, whereas the filamentous phage can easily accommodate polypeptides the size of an antibody chain (Soderlind et al., 1992).

(e) Conclusions

Although only incomplete libraries (500 000 beads each) were screened in these studies, several important conclusions can still be drawn: (*i*) D-aa containing ligands can be isolated from a random synthetic peptide library, (*ii*) the anti-βEN mAb can accept mimotopes with D-aa at specific positions although with lower binding affinities, (*iii*) studies described here can provide valuable data that otherwise would be extremely difficult to obtain by means other than the synthetic peptide library approach, and (*iv*) these data potentially can lead us to a better understanding of peptide-antibody interaction, and the role of chirality in molecular recognition.

In addition to anti-βEN mAb, we have also screened similar D-aa-containing peptide libraries with streptavidin and have discovered several motifs of D-aa-containing

ligands that interact specifically with streptavidin but not with avidin, and the results will be reported elsewhere (Lam et al., 1994a). Using an anti-insulin murine mAb as a molecular target, we have also discovered various L-aa (Lebl et al., 1993) and D-aa-containing peptide motifs that mimic a discontinuous epitope on human insulin. However, unlike the D-aa-containing ligands that are described here for the anti-βEN mAb, the primary sequences of the D-aa-containing ligands for anti-insulin mAb showed no resemblance with that of the L-ligands (Lam et al., 1994b). Work is now underway in our laboratories to use various macromolecular targets to screen peptide libraries with other unnatural aa, cyclic peptide libraries, and non-peptide chemical libraries (Nikolaiev et al., 1993).

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