We developed an expression cloning method for identifying novel peptide agonists based on the thrombin receptor’s activation mechanism. Thrombin cleaves its receptor’s amino-terminal exodomain to unmask a new amino terminus, which then functions as a tethered peptide agonist, binding intramolecularly to the body of the receptor to trigger signaling. The thrombin receptor’s amino-terminal exodomain can also donate the tethered agonist intermolecularly to activate nearby thrombin receptors. We utilized this ability by co-expressing a “tethered ligand library,” which displayed the thrombin receptor’s amino-terminal exodomain bearing random pentapeptides in place of the native tethered ligand together with target receptors in Xenopus oocytes. Clones that conferred thrombin-dependent signaling by intermolecular ligation of the target receptor were isolated by sib selection. Agonists for the thrombin receptor itself (GFIYF) and for the formyl peptide receptor (MMWLL) were identified. Surprisingly, the latter agonist was quite active at the formyl peptide receptor even without N-formylation, and its formylated form, fMMWLL, was more potent than the classical formyl peptide receptor agonist fMLF. Peptides mimicking the tethered ligand sequences of these clones, GFIYF for thrombin receptor agonist and MMWLL for formyl peptide receptor, were effective agonists for their respective targets. The expected and unexpected features of these agonists are discussed. The tethered ligand method thus provided an approach for searching a diverse group of peptide sequences for agonist activity. In addition to identifying novel peptide agonists for targets of pharmacological interest, this method might be used to discover agonists for orphan receptors. The ease with which agonists were “evolved” in this system suggests a possible evolutionary path from peptide to protease-activated receptors.

**EXPERIMENTAL PROCEDURES**

Xenopus oocytes were harvested, microinjected with cRNAs, and cultured as previously described (1). Agonist-induced responses were assessed as [Ca\(^{2+}\)]\(_i\) release (1).

The library was constructed as follows. A construct dubbed ATE-CD8 encoding the thrombin receptor’s amino-terminal exodomain (ATE),\(^1\) fused to the transmembrane domain of CD8, was used to display the receptor’s amino-terminal exodomain on the cell surface (2). Surface expression of the encoded protein and its cleavage by thrombin was verified as the presence and thrombin-dependent loss of an epitope placed amino to the thrombin cleavage site (2–4). A library of such molecules in which the receptor’s agonist peptide domain SFLLR was replaced by random pentapeptides was constructed as follows. ATE-CD8 was modified to introduce paired BstXI sites flanking the agonist peptide domain so that a “BstXI cassette” could later be inserted to make the library (5, 6). An in-frame nonsense mutation was inserted between these sites to ensure premature termination of translation of any non-recombinant ATE-CD8 molecules occurring in the library. The modified ATE-CD8 was inserted into pBLOG between Xenopus globin mRNA 5’- and 3’-untranslated sequence and downstream of an SP6 RNA polymerase promoter. pBLOG was made by subcloning a 0.9-kilobase EcoRI-PstI fragment from pFROG (1) into the corresponding sites of pBluescript II SK\(^{-}\) (Stratagene) in which the BstXI site had been destroyed. BstXI cassette inserts for the library were generated by annealing a pool of degenerate oligonucleotides S-GATCCCCGGGNKAAAACCACACTGATATAATGACCCATT-3’, where N = A, C, G, or T in equal molar amounts and K = G or T in equal molar amounts with two complementary, flanking 13 mers 5’-CCGGGGATCTAAAG-3’ and 5’-GTTCATATCTTAC-3’ (7). The annealed oligomers were then ligated into the new BstXI sites of the modified ATE-CD8 in pBLOG. Escherichia coli strain DH10B was electroporated with an aliquot of the resulting library and plated at a complexity of 4000/pool. Of 10 randomly picked clones that were sequenced (8), 8

\(^1\) The abbreviation used is: ATE, amino-terminal exodomain.
tethered ligand library selection "evolved" an agonist that shares critical features with the known naturally occurring thrombin receptor tethered ligand sequences. A unique feature of the GFIYF agonist, however, is its lack of a basic residue. All of the known tethered ligand sequences sport an arginine at either position 3 or 5, raising the possibility that these arginines serve functions beyond receptor activation in the native receptor and its cellular context.

Can this technique be used to find agonists for receptors that are not naturally activated by a tethered ligand mechanism? To test this question, we co-expressed pools representing 10,000 independent tethered ligand library clones with a variety of potential targets: the SHT1C (11), β2-adrenergic (12), interleukin-8 (13), MCP-1 (14), formyl peptide (15), or RTA (16) receptors. A pool with agonist activity for the formyl peptide receptor was immediately identified even at this relatively low level of complexity. We subsequently screened 11 subpools of 800 independent clones each for activity at the formyl peptide receptor. The most active of these was serially subdivided, and a single cDNA that conferred thrombin-dependent signaling was isolated by sib selection.

The predicted amino acid sequence of this clone's tethered ligand domain was GFIYF. A synthetic peptide mimicking this domain was nearly as effective as the SFLLR peptide, which mimics the native tethered ligand sequence (Fig. 3A). Moreover, a mutant thrombin receptor in which the GFIYF sequence replaced the native SFLLR sequence signaled as effectively as the wt receptor in response to thrombin (Fig. 3B).

The sequence of this new agonist is striking in the context of known thrombin receptor agonist peptide sequences and structure-activity relationships (Table 1). The protonated amino group at the amino-terminal of all thrombin receptor peptide agonists (the group corresponding to that created by receptor cleavage) is critical for agonist function, and small neutral residues are preferred at agonist position 1. The conserved phenylalanine at position 2 is critical for agonist function (9, 10). Hydrophobic residues are preferred at positions 3 and 4. All of these features are captured in GFIYF. Moreover, the phenylalanine at position 5 in the GFIYF peptide is seen in the Xenopus thrombin receptor's tethered ligand sequence. Thus, indeed encoded a wild-type ATE-CD8 sequence except for the 15 nucleotides encoding the random agonist pentapeptide; 2 had mutations probably arising during annealing or ligation. Thus, approximately 80% of the library encoded molecules that would display random pentapeptides at their amino termini upon cleavage by thrombin. The library was co-expressed with target receptors in Xenopus oocytes by co-injecting cRNAs (See Fig. 2).

The predicted amino acid sequence of this clone's tethered ligand domain was GFIYF. A synthetic peptide mimicking this domain was nearly as effective as the SFLLR peptide, which mimics the native tethered ligand sequence (indicated by the various shapes) so that cleavage of the library by thrombin would unmask a set of potential tethered peptide agonists. When co-expressed with a target receptor in Xenopus oocytes, a member of the library bearing an appropriate tethered ligand sequence (open diamond) intermolecularly ligands and activates the target receptor. Functionally this is seen as thrombin-triggered signaling.

FIG. 1. Tethered ligand library design. The library utilized the thrombin receptor's amino-terminal exodomain fused to a transmembrane domain to display potential tethered agonists on the oocyte surface. The thrombin receptor's native agonist peptide SFLLR was replaced with random pentapeptide sequence (indicated by the various shapes) so that cleavage of the library by thrombin would unmask a set of potential tethered peptide agonists. When co-expressed with a target receptor in Xenopus oocytes, a member of the library bearing an appropriate tethered ligand sequence (open diamond) intermolecularly ligands and activates the target receptor. Functionally this is seen as thrombin-triggered signaling.

FIG. 2. Thrombin-induced 45Ca release from oocytes co-expressing the first set of library pools and the F43A mutant thrombin receptor. cRNA was transcribed from cDNA representing pools of 4000 independent clones (1). Xenopus oocytes were co-injected with 25 ng each of library and F43A mutant thrombin receptor cRNAs and cultured for 24 h, after which thrombin-stimulated 45Ca release was assessed (1). Results (mean ± S.D. (n = 3)) are expressed as 45Ca release in the 10 min following addition of 20 nm thrombin (baseline 45Ca release in the preceding 10 min (1). The high concentration of thrombin (20 nm) was used in an effort to avoid selecting clones based on their substrate properties. Oocytes expressing the library or F43A alone showed no signaling to thrombin (not shown). Co-expression resulted in clear-cut thrombin signaling with three of the nine pools tested. A clone was isolated from the most active of these (pool 9, arrow) by sib selection (1).

RESULTS AND DISCUSSION

Pools of the tethered ligand library each representing 4000 independent clones (Fig. 1) were co-expressed with a target thrombin receptor bearing the F43A loss of function mutation in its tethered ligand domain (2, 9, 10). This receptor could be activated by free synthetic agonist peptide but, lacking its own functional tethered ligand, could not be activated by thrombin (2). Signaling in response to thrombin in the co-expression system thus reflected intermolecular ligation of the target receptor by a member of the library. Of the first 9 pools tested, 3 clearly conferred thrombin-dependent signaling when co-expressed with the F43A receptor (Fig. 2). From the most active of these pools, a single clone that conferred signaling was isolated by sib selection.

The predicted amino acid sequence of this clone's tethered ligand domain was GFIYF. A synthetic peptide mimicking this domain was nearly as effective as the SFLLR peptide, which mimics the native tethered ligand sequence (indicated by the various shapes) so that cleavage of the library by thrombin would unmask a set of potential tethered peptide agonists. When co-expressed with a target receptor in Xenopus oocytes, a member of the library bearing an appropriate tethered ligand sequence (open diamond) intermolecularly ligands and activates the target receptor. Functionally this is seen as thrombin-triggered signaling.
These data suggest that the formyl peptide receptor, contains sufficient information by itself to function as an agonist for activating the formyl peptide receptor, but MMWLL is 1000-fold more potent than fMLF itself (Fig. 4). Thus, the amino-terminal formyl group probably provides new interactions important for activating the formyl peptide receptor, but MMWLL contains sufficient information by itself to function as an agonist. These data suggest that the formyl peptide receptor, thought to be important for mediating chemotaxis toward N-formylated bacterial proteins, remains competent to respond to certain unformylated peptides and may have evolved from a pre-existing peptide receptor. Whether endogenous unformylated peptide agonists for this receptor exist is unknown.

The ease with which agonists were identified using the tethered ligand library prompts speculation regarding the evolutionary origin of protease-activated receptors such as the thrombin receptor and protease-activated receptor 2 (20). Of 3.2 × 10^6 possible tethered ligand sequences in the library, only 36,000 were screened to find a thrombin receptor agonist and 10,000 to find a formyl peptide receptor agonist. From the prevalence of positive pools in the library, we estimate that at least 1 in 10,000 tethered ligand sequences had some agonist activity at the thrombin receptor or the fMLF receptor. The intermolecular liganding relied upon in these screens is much less efficient than the intramolecular liganding that mediates activation of native protease receptors (2). Moreover, the tethered ligand in the intact receptor is quite tolerant of amino acid substitutions; alanine substitution at agonist positions 3, 4, and 5 cause loss of function in the free agonist peptide but not the intact receptor (1, 10, 21). Because of the kinetic advantages conferred by tethering, sequences capable of functioning as tethered ligands may have appeared relatively frequently in the amino-terminal exodomain of peptide receptors during their evolution. An early protease-activated receptor may thus have arisen from a pre-existing peptide receptor when mutation created a protease cleavage site amino-terminal to a potential tethered ligand sequence.

In summary, the tethered ligand library method has identified new agonists for two “peptide” receptors. The new agonists revealed both expected and unexpected features. The tethering feature of this method provides an advantage over use of free peptides for identifying low affinity leads, and use of signaling as a readout selects for agonists over antagonists. The extent to which this technique can be applied to other G protein-coupled receptors and signaling molecules remains to be determined. Adaptation of this approach to yeast (22) or mammalian cell systems (23) that provide functional readouts for receptor activation may broaden the applicability of this technique by providing higher throughputs. In principle, the tethered ligand library provides a rapid means of establishing consensus sequences for peptide agonists to aid structure-activity studies as well as a method for identifying agonists for orphan receptors.

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