Anionic Amino Acids near the Pro-α-defensin N Terminus Mediate Inhibition of Bactericidal Activity in Mouse Pro-cryptdin-4

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In mouse Paneth cells, α-defensins, termed cryptdins (Crps), are activated by matrix metalloproteinase-7–mediated proteolysis of inactive precursors (pro-Crps) to bactericidal forms. The activating cleavage step at Ser43Ile in mouse pro-Crps (20–92) removes nine acidic amino acids that collectively block the membrane-disruptive behavior of the Crp4 moiety of the proform. This inhibitory mechanism has been investigated further to identify whether specific cluster(s) of electronegative amino acids in pro-Crps (20–43) are responsible for blocking bactericidal activity and membrane disruption. To test whether specific cluster(s) of electronegative amino acids in pro-Crps (20–43) have specific positional effects that block bactericidal peptide activity and membrane disruption, acidic residues positioned at the distal (Asp20, Asp26, Glu27, and Glu28), mid (Glu32 and Glu33), and proximal (Glu37, Glu38, and Asp39) clusters in pro-Crps (20–92) were mutagenized, and variants were assayed for differential effects of mutagenesis on bactericidal peptide activity. Substitution of the mid and proximal Asp and Glu clusters with Gly or their deletion resulted in the crypt lumen is inferred to protect mitotically active crypt cells from colonization by potential pathogens and to confer protection from enteric infection (2, 8–10). The most compelling evidence for a Paneth cell role in enteric innate immunity is evident from studies of mice transgenic for a human Paneth cell α-defensin, HD-5, which are completely immune to infection and systemic disease from orally administered Salmonella enterica serovar typhimurium (11).

The biosynthesis of α-defensins requires post-translational activation by lineage-specific proteases (12, 13). Although the enzymes that mediate pro-α-defensin processing in myeloid and epithelial cells differ, the overall processing schemes are the same. Both myeloid and Paneth cell α-defensins derive from ~10-kDa prepropeptides that contain canonical signal sequences, electronegative proregions, and a 3.5–4-kDa mature α-defensin peptide in the C-terminal portion of the precursor (13–16). Pro-α-defensin processing in mouse Paneth cells is catalyzed by matrix metalloproteinase-7 (MMP-7)3 and takes place intracellularly and prior to secretion (17, 18). In mouse small intestinal epithelium, only Paneth cells express MMP-7 as components of dense core secretory granules (12), and the bactericidal activity of mouse Paneth cell α-defensins depends completely on activation of 8.4-kDa pro-Crps by MMP-7-catalyzed proteolysis (12, 18). MMP-7 gene disruption ablates pro-Crp processing such that mature, activated Crp peptides are absent from the small intestine, and innate immunity to oral bacterial infection is impaired in MMP-7-null mice (12).

In the small bowel, Paneth cells at the base of the crypts of Lieberkühn secrete α-defensins and additional antimicrobial peptides at high levels in response to cholinergic stimulation and when exposed to bacterial antigens (1–4). Paneth cell α-defensins show broad spectrum antimicrobial activities and constitute the majority of bactericidal peptide activity in Paneth cell secretions (2, 5–7). The release of Paneth cell products into the crypt lumen is inferred to protect mitotically active crypt cells from colonization by potential pathogens and to confer protection from enteric infection (2, 8–10). The most compelling evidence for a Paneth cell role in enteric innate immunity is evident from studies of mice transgenic for a human Paneth cell α-defensin, HD-5, which are completely immune to infection and systemic disease from orally administered Salmonella enterica serovar typhimurium (11).

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3 The abbreviations used are: MMP-7, matrix metalloproteinase-7; Crp4, cryptdin-4; pro-Crps, pro-cryptdins; HD-5, mucosal defensin-5; ONPG, 2-nitrophenyl-β-D-galactopyranoside; HPLC, high performance liquid chromatography; AU-PAGE, acid/urea-polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TSB, Trypticase soy broth; PIPES, 1,4-piperazinediethanesulfonic acid; CFU, colony-forming units.
N-terminal Acids Amino Acids Block Pro-Crp4 Activity

MMP-7 produces active mouse α-defensins by cleaving precursors in vitro at conserved sites in the proregion including the junction of the propeptide and α-defensin moiety (18). Electropositive Arg side chains in Crp4 facilitate electrostatic interactions necessary for disruption of the electronegative bacterial cell envelope (3, 19, 20). In contrast, pro-Crps lack in vitro bactericidal activity, corresponding with a diminished ability to interact with model membranes, to disrupt large unilamellar vesicles (20), and to permeabilize live cells. MMP-7-mediated proteolysis of pro-Crp4-(20–92) prepared by site-directed mutagenesis (18), expressed as N-terminal His6-tagged fusion proteins, and subsequently affinity-purified, results in the specific cleavage intermediates pro-Crp4-(44–92), pro-Crp4-(54–92), and pro-Crp4-(59–92) (18). Because the primary ONP production are dependent on peptide-mediated permeabilization assays. The results show that the acidic residues nearest the N-terminal Asp of native pro-Crp4-(20–92).

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Peptides—Recombinant pro-Crp4-(20–92) preregion variants were prepared by site-directed PCR-based mutagenesis (18), expressed as N-terminal His6-tagged fusion proteins, and subsequently affinity-purified (3, 18, 22). After cleavage of the His6 tag, the peptides were further purified by HPLC. Peptide homogeneity was assessed by analytical reverse-phase HPLC and acid/urea (AU)-PAGE (23), and peptide masses were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE) (21). The experimental details of mutagenesis and preparation of the individual recombinant peptides investigated (Fig. 1) are described in detail (see supplemental “Experimental Procedures”).

MMP-7-mediated Proteolysis of Recombinant Peptides—To test pro-Crp4 variants for proteolytic conversion by MMP-7, 10-μg peptide samples were incubated with or without 0.5 mol eq of MMP-7 in HEPES buffer (1 mM HEPES, 15 mM NaCl, 0.5 mM CaCl2, pH 7.4) for 18 h at 37 °C. Complete peptide digests were resolved by AU-PAGE and stained with Coomassie Blue.

Bactericidal Peptide Assays—The bactericidal activities of purified recombinant peptides were tested in vitro against E. coli ML35, Staphylococcus aureus 502A, Vibrio cholerae, Listeria monocytogenes 104035, and S. enterica serovar typhimurium strains as described previously (21, 24). Samples consisting of exponentially growing bacterial cells were collected by centrifugation, washed, and resuspended in 10 mM PIPES, pH 7.4, supplemented with 0.01 volume (1%, v/v) of Trypticase soy broth (PIPES/TSB). Bacteria (5 × 10^6 colony-forming units (CFU)/ml) were incubated with each peptide (0–6 μM) for 12–18 h, and data were analyzed and plotted using SigmaPlot (Systat Software, Inc., San Jose, CA). Bactericidal assays performed in Figs. 3, 4, and 7 are representative of experiments that are reliably reproducible (see supplemental Fig. S4).
brane disruption. ONP was measured at 405 nm on a 96-well SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA), and data were analyzed using SigmaPlot.

RESULTS

MMP-7 Processes Pro-Crp4 Anionic Proregion Variant Peptides—To test whether partial reduction of the electronegative charge in the proregion of pro-Crp4 has effects on procursor folding and processing, the native and variant pro-Crp4 peptides were incubated with MMP-7 and analyzed by AU-PAGE. Pro-Crp4 requires MMP-7-mediated proteolytic conversion to Crp4, and disruption of disulfide pairings would result in extensive peptide proteolysis (22). Because properly folded Crp4 resists proteolysis completely and Crp4 and its fragments do not stain with Coomassie Blue.

Effects of Partial Electronegative Charge Neutralization on Pro-Crp4 Bactericidal Peptide Activity—To determine whether partial reduction of anionic charge in pro-Crp4-(20–43) exerts differential effects on the inhibition of pro-Crp4-(20–92) bactericidal activity, the proregion variants of pro-Crp4 were tested against S. aureus 502A, L. monocytogenes 104035, E. coli ML35, V. cholerae, and S. enterica serovar typhimurium strains including 14028s, CS022, and ΔphoP (3). Native pro-Crp4-(20–92), which lacks activity, served as a negative control peptide with bactericidal Crp4 and (DE/G)-pro-Crp4 molecules serving as positive controls (18, 21).

To test whether the bactericidal activity of (DE/G)-pro-Crp4 resulted from conferring activity on the proregion per se by mutagenesis of the electronegative residues to glycines, the mutant proregion (DE/G)-pro-Crp4-(20–60) also was assayed for microbicidal activity (see “Experimental Procedures”). In all instances, (DE/G)-pro-Crp4-(20–60) had no effect on bacterial cell survival up to 6 μM peptide concentration (Fig. 3, A–F). These findings show that the activity of (DE/G)-pro-Crp4-(20–92) is due to the proregion loss of inhibitory function and is not a consequence of (DE/G)-pro-Crp4-(20–60) being converted to a bactericidal molecule as a result of the mutagenesis.

To test the hypothesis that removal of carboxyl groups alone at Asp and Glu positions would be sufficient to eliminate proregion inhibitory effects, we prepared (DE/NQ)-pro-Crp4-(20–92) in which Asn and Gln residue positions would retain side chain length but lack anionic charge. Against the less sensitive S. aureus and S. enterica serovar typhimurium CS022 and 14028s species, even 6 μM (DE/NQ)-pro-Crp4 showed very lit-
N-terminal Acidic Amino Acids Block Pro-Crp4 Activity

To test the hypothesis that individual clusters of anionic amino acids in pro-Crp4-(20–43) exert differential effects in blocking pro-Crp4 bactericidal activity, the peptide activities of pro-Crp4 variants with partially reduced anionic charge in the distal (Asp20, Asp26, Glu27, and Glu28), mid (Glu32 and Glu33), and side chain interactions that were not evident in studies of combination of charge neutralization of Crp4 cationic residues appear to inhibit pro-Crp4-(20–92) bactericidal activity by a combination of charge neutralization of Crp4 cationic residues and side chain interactions that were not evident in studies of (DE/G)-pro-Crp4.

To test the hypothesis that individual clusters of anionic amino acids in pro-Crp4-(20–43) exert differential effects in blocking pro-Crp4 bactericidal activity, the peptide activities of pro-Crp4 variants with partially reduced anionic charge in the distal (Asp20, Asp26, Glu27, and Glu28), mid (Glu32 and Glu33), and proximal (Glu34, Glu35, and Asp36) anionic clusters in pro-Crp4-(20–92) were compared with pro-Crp4-(20–92) (Fig. 1). Peptides with combined charge neutralization of the distal and proximal clusters, (6-DE/G)-pro-Crp4, and of the mid and proximal clusters, (5-DE/G)-pro-Crp4, were more active than pro-Crp4 but not as active as (DE/G)-pro-Crp4 or Crp4 (Fig. 3, A–F). The proximal pro-Crp4 variant (3-DE/G)-pro-Crp4 was attenuated compared with (6-DE/G)-pro-Crp4 and (5-DE/G)-pro-Crp4, suggesting that anionic clusters contribute to blocking pro-Crp4 activity in an additive manner (Fig. 4, A–D). However, mutagenesis of the distal anionic amino acid cluster showed a markedly greater and differential effect on pro-Crp4 bactericidal activity as compared with mid and proximal cluster charge neutralization (Fig. 4).

**Mutagenesis of Anionic Amino Acid Residues in Proregion of Pro-Crp4 Enables Permeabilization of Live E. coli ML35 Cells**—To test the hypothesis that individual clusters of anionic amino acids in pro-Crp4-(20–43) exert differential effects on defensin-microbe interactions and permeabilization of live bacteria, the pro-Crp4 variant peptides were compared with Crp4 and pro-Crp4 using the ONPG conversion assay (see “Experimental Procedures”). Consistent with their lack of bactericidal peptide activity, (DE/G)-pro-Crp4-(20–60) and pro-Crp4-(20–92) did not permeabilize E. coli (see supplemental Fig. S2). Relative to the effects of Crp4, bacterial exposure to 3 μM (DE/G)- and (DE/NQ)-pro-Crp4 resulted in a delay in measurable ONP accumulation (Fig. 5). In fact, at 3 μM, no ONP accumulation was detected as a result of exposure to pro-Crp4 variant peptides, suggesting an attenuation of membrane-disruptive activity for the partial electronegative variants (see also supplemental Fig. S3). On the other hand, at 6 μM, comparison of pro-Crp4 peptides with a combined charge neutralization of distal and proximal clusters or with only the proximal cluster neutralized supported the view that the anionic clusters provided an additive inhibitory effect on pro-Crp4-induced cell permeabilization (Fig. 6A). Deletion of the distal cluster conferred permeabilizing activity, but the induced ONPG conversion was not as robust as that caused by mature Crp4 (Fig. 6B).

**The Most N-terminal Electronegative Cluster Is Primarily Responsible for Maintaining Pro-Crp4 in an Inhibited State**—Acidic amino acid residue positions in the most distal proregion cluster predominate in inhibiting pro-Crp4 bactericidal activity. For example, against S. enterica serovar typhimurium ΔphoP, L. monocytogenes, and E. coli, collective mutagenesis of the proximal and distal clusters (6-DE/G)-pro-Crp4 increased peptide activity to a greater extent than mutagenesis at the proximal cluster alone (3-DE/G)-pro-Crp4 (Fig. 3, A, B, and F). Mutagenesis of the distal cluster alone improved bactericidal activity much more than the combined mutagenesis of mid and proximal clusters, as evident in assays against S. enterica serovar typhimurium ΔphoP, L. monocytogenes, and E. coli (Fig. 4, A–C). For reasons that remain unclear, the recoveries of distal cluster variant (4-DE/G)-pro-Crp4 and combined proximal and mid cluster mutant (5-DE/G)-pro-Crp4 were unusually low, requiring that their bactericidal peptide activities be tested in separate experiments (Fig. 4, A–D). Remarkably, mutagenesis of the distal cluster resulted in (4-DE/G)-pro-Crp4 being as or more active than mature Crp4 at
concentrations $\geq 1.5$ $\mu M$, and the distal cluster mutant was more bactericidal than any partial charge neutralization mutants (Fig. 4A). To summarize, the relative bactericidal activities of all partial anionic pro-Crp4 variants are as follows: Crp4 = (DE/G)-pro-Crp4 = (4-DE/G)-pro-Crp4 > (6-DE/G)-pro-Crp4 = (5-DE/G)-pro-Crp4 > (3-DE/G)-pro-Crp4 > pro-Crp4. Thus, the acidic residues in the distal cluster of the proregion inhibit pro-Crp4 differentially.

Deletion of Distal Anionic Residue Cluster Confirms Its Inhibitory Role—To test the role of the distal acidic amino acid cluster further, a variant peptide, pro-Crp4-(29–92), with a deletion of the nine N-terminal amino acids of pro-Crp4-(20–92) was studied (Fig. 7, A–F). As observed for charge neutralization of the distal cluster in (4-DE/G)-pro-Crp4, deletion of the distal anionic cluster resulted in the peptide having equivalent or better activity than Crp4 (Fig. 7, A–C and F). Thus, whether the distal cluster was modified by charge neutralization or by deletion, the results confirmed that the most N-terminal acidic residue positions are mainly responsible for maintaining pro-Crp4-(20–92) in an inactive state.

**DISCUSSION**

This investigation tested whether specific cluster(s) of electronegative amino acids in pro-Crp4-(20–43) have specific positional effects that block bactericidal peptide activity and membrane disruption. Acidic residues positioned at the distal (Asp$^{20}$, Asp$^{26}$, Glu$^{27}$, and Glu$^{28}$), mid (Glu$^{32}$ and Glu$^{33}$), and proximal (Glu$^{37}$, Glu$^{38}$, and Asp$^{39}$) clusters in pro-Crp4-(20–92) were mutagenized, and variants were assayed for differential effects of mutagenesis on bactericidal peptide activity. Certain substitutions produced additive effects on the induction of both bactericidal activity and permeabilization of live *E. coli* ML35 cells. In contrast, substitution of distal Glu and Asp residues with Gly or their deletion resulted in variants with bactericidal and membrane-disruptive activities equivalent to or greater than those of the fully mature Crp4 peptide. These findings support the conclusion that the distal anionic residues near the N terminus of pro-Crp4-(20–43) are primarily responsible for blocking precursor bactericidal action. Because (DE/NQ)-pro-Crp4 has intermediate bactericidal activity (Fig. 3), we cannot exclude the possibility that proregion-Crp4 side chain interactions that are not charge-neutralizing also contribute to blocking pro-Crp4 activity. Although the effect of neutralizing different anionic amino acid clusters suggested that the clusters exert additive inhibitory
effects (Figs. 3 and 4), mutagenesis of the distal anionic amino acid residues alone, *i.e.* in (4-DE/G)-pro-Crp4 and pro-Crp4-(29–92), supports a greater role for residues positioned nearest the pro-Crp-(20–92) N terminus.

Individual pro-α-defensins may have evolved differing mechanisms to achieve the same inhibitory effect as those described for pro-Crp4. For example, in the human neutrophil α-defensin precursor pro-HNP-1, interactions between hydrophobic proregion residues with those in the HNP-1 moiety appear to be critical to maintaining pro-HNP-1 in an inactive state (30). Furthermore, in striking contrast to the findings we report, pro-HNP-1 remained inactive following deletion of nine amino acids from the pro-HNP-1 N terminus, including three acidic residues, showing that they are functionally dispensable in this respect (30). Thus, the role(s) of individual proregion residue positions in maintaining pro-α-defensin inhibition vary, possibly depending on the distribution of electronegative charge or the orientation of peptide amphipathicity along the α-defensin triple-stranded β-sheet topology. Clearly, these features vary with peptide primary structure in that all α-defensins share a common constrained tertiary structure. In this respect, Crp4 and RMAD-4 (rhesus myeloid α-defensin-4) differ markedly with regard to charge distribution and the orientation of hydrophobic side chains along the peptide fold. Still, neutralizing anionic amino acids in the proregion of pro-RMAD-4-(20–94) have the same activating effects as those described here for pro-Crp4-(20–92) (36). Possibly, the biochemistry of the HNP precursor may have a unique structural context, leaving the question open until additional molecules appear to be critical to maintaining pro-HNP-1 in an inactive state during biosynthesis and trafficking may be critical to the viability of both promyelocytes and Paneth cells, lineages that express these innate immune effector molecules at very high levels.

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4 H. J. Vogel, personal communication.

REFERENCES

1. Selsted, M. E., and Ouellette, A. J. (2005) *Nat. Immunol.* **6**, 551–557
2. Ouellette, A. J. (2005) *Springer Semin. Immunopathol.* **27**, 133–146
3. Tanabe, H., Qu, X., Weeks, C. S., Cummings, J. E., Kolusheva, S., Walsh, K. B., Jelinek, R., Vanderlick, T. K., Selsted, M. E., and Ouellette, A. J. (2004) *J. Biol. Chem.* **279**, 11976–11983
4. Ouellette, A. J. (1999) *Am. J. Physiol.* **277**, G257–G261
5. Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., and Ouellette, A. J. (2000) *Nat. Immunol.* **1**, 113–118
6. Porter, E. M., Bevins, C. L., Ghosh, D., and Ganz, T. (2002) *CMLS* **59**, 156–170
7. Zasloff, M. (2002) *Nature* **415**, 389–395
8. Ouellette, A. J., and Bevins, C. L. (2005) in *Antimicrobial Peptides in Human Health and Disease* (Gallo, R. L., ed) pp. 175–198, Horizon Scientific Press, Norfolk, UK
9. Ganz, T. (2003) *Nat. Rev. Immunol.* **3**, 710–720
10. Ganz, T. (2004) *C. R. Biol.* **327**, 539–549
11. Salzman, N. H., Ghosh, D., Hutten, K. M., Paterson, Y., and Bevins, C. L. (2003) *Nature* **422**, 522–526
12. Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999) *Science* **286**, 113–117
13. Ghosh, D., Porter, E., Shen, B., Lee, S. K., Wilk, D., Drabza, J., Yadav, S. P., Crab, J. W., Ganz, T., and Bevins, C. L. (2002) *Nat. Immunol.* **3**, 583–590
14. Ganz, T., Liu, L., Valore, E. V., and Oren, A. (1993) *Blood* **82**, 641–650
15. Michaelson, D., Rayner, J., Courto, M., and Ganz, T. (1992) *J. Leukocyte Biol.* **51**, 634–639
16. Valore, E. V., and Ganz, T. (1992) *Blood* **79**, 1538–1544
17. Ayabe, T., Satchell, D. P., Pesendorfer, P., Tanabe, H., Wilson, C. L., Ha- gen, S. J., and Ouellette, A. J. (2002) *J. Biol. Chem.* **277**, 5219–5228
18. Shiraufi, Y., Tanabe, H., Satchell, D. P., Henschke-Edman, A., Wilson, C. L., and Ouellette, A. J. (2003) *J. Biol. Chem.* **278**, 7910–7919
19. Satchell, D. P., Sheynis, T., Kolusheva, S., Cummings, J., Vanderlick, T. K., Jelinek, R., Selsted, M. E., and Ouellette, A. J. (2003) *Peptides (Elmsford)* **24**, 1795–1805
20. Satchell, D. P., Sheynis, T., Shiraufi, Y., Kolusheva, S., Ouellette, A. J., and Selsted, R. E. (2003) *J. Biol. Chem.* **278**, 13838–13846
21. Weeks, C. S., Tanabe, H., Cummings, J. E., Crampton, S. P., Sheynis, T., Jelinek, R., Vanderlick, T. K., Cocco, M. I., and Ouellette, A. J. (2006) *J. Biol. Chem.* **281**, 28932–28942
22. Maemoto, A., Qu, X., Rosenwegen, K. J., Tanabe, H., Henschke-Edman, A., Craik, D. I., and Ouellette, A. J. (2004) *J. Biol. Chem.* **279**, 44188–44196
23. Selsted, M. E. (1993) in *Genetic Engineering: Principles and Methods* (Setlow, J. K., ed) pp. 131–147, Plenum Press, New York
24. Jing, W., Hunter, H. N., Tanabe, H., Ouellette, A. J., and Vogel, H. J. (2003) *Biochemistry* **43**, 15759–15766
25. Ayabe, T., Wulfh, D., Darmoul, D., Cahalan, M. D., Chandy, K. G., and Ouellette, A. J. (2002) *J. Biol. Chem.* **277**, 3793–3800
26. Matsuoka, K., Mitani, Y., Akada, K. Y., Murase, O., Yoneyma, S., Zasloff, M., and Miyajima, K. (1998) *Biochimica Biochemica* **37**, 15144–15153
27. Zanetti, M., Gennaro, R., and Romeo, D. (1997) *Ann. N. Y. Acad. Sci.* **832**, 147–162
28. White, S. H., Wimley, W. C., and Selsted, M. E. (1995) *Curr. Opin. Struct. Biol.* **5**, 521–527
29. Hristova, K., Selsted, M. E., and White, S. H. (1997) *J. Biol. Chem.* **272**, 24224–24233
30. Zou, G., de Leeuw, E., Lubkowsi, J., and Lu, W. (2008) *J. Mol. Biol.* **381**, 1281–1291
31. Liu, L., and Ganz, T. (1995) *Blood* **85**, 1095–1103
32. Wu, Z., Li, X., Ericksen, B., de Leeuw, E., Zou, G., Zeng, P., Xie, C., Li, C., Lubkowsi, J., Lu, W. Y., and Lu, W. (2007) *J. Mol. Biol.* **368**, 537–549
33. Walter, P., and Johnson, A. E. (1994) *Annu. Rev. Cell Biol.* **10**, 87–119
34. Kuliyavat, R., and Arvan, P. (1992) *J. Cell Biol.* **118**, 521–529
35. Traub, L. M., and Kornfeld, S. (1997) *Curr. Opin. Cell Biol.* **9**, 527–533
36. Kamdar, K., Maemoto, A., Qu, X., Young, S. K., and Ouellette, A. J. (2008) *J. Biol. Chem.* **283**, 32361–32368