

Transglutaminase-catalyzed incorporation of polyamines masks the DNA-binding region of the transcription factor Relish

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In *Drosophila*, the final immune deficiency (IMD) pathway-dependent signal is transmitted through proteolytic conversion of the nuclear factor- κ B (NF- κ B)-like transcription factor Relish to the active N-terminal fragment Relish-N. Relish-N is then translocated from the cytosol into the nucleus for the expression of IMD-controlled genes. We previously demonstrated that transglutaminase (TG) suppresses the IMD pathway by polymerizing Relish-N to inhibit its nuclear translocation. Conversely, we also demonstrated that orally ingested synthetic amines, such as monodansylcadaverine (DCA) and biotin-labeled pentylamine, are TG-dependently incorporated into Relish-N, causing the nuclear translocation of modified Relish-N in gut epithelial cells. It remains unclear, however, whether polyamine-containing Relish-N retains transcriptional activity. Here, we used mass spectrometry analysis of a recombinant Relish-N modified with DCA by TG activity after proteolytic digestion and show that the DCA-modified Gln residues are located in the DNA-binding region of Relish-N. TG-catalyzed DCA incorporation inhibited binding of Relish-N to the Rel-responsive element in the NF- κ B-binding DNA sequence. Subcellular fractionation of TG-expressing *Drosophila* S2 cells indicated that TG was localized in both the cytosol and nucleus. Of note, natural polyamines, including spermidine and spermine, competitively inhibited TG-dependent DCA incorporation into Relish-N. Moreover, *in vivo* experiments demonstrated that Relish-N was modified by spermine and that this modification reduced transcription of IMD pathway-controlled *cecropin A1* and *diptericin* genes. These findings suggest that intracellular TG regulates Relish-N-mediated transcriptional activity by incorporating polyamines into Relish-N and via protein-protein cross-linking.

Protein-protein cross-linking catalyzed by transglutaminase (TG)³ plays important and diverse roles in various physiologic phenomena, such as blood coagulation, skin barrier formation, and apoptosis (1). TG catalyzes the isopeptide formation of ϵ -(γ -glutamyl) lysine bonds in a Ca²⁺-dependent manner (2). TGs function both intracellularly and extracellularly, and many TG substrates have been identified in metazoans (3–5). The mammalian genome contains eight encoded isoenzymes with each member exhibiting diverse functions: factor XIIIa is essential for blood coagulation, and TG2 is involved in apoptosis, cell adhesion, and inflammatory responses (1). In invertebrates, such as the horseshoe crab *Tachypleus tridentatus*, the crayfish *Pacifastacus leniusculus*, and the fruit fly *Drosophila melanogaster*, TGs also exhibit pleiotropic functions, including hemolymph coagulation, cuticle formation, and immobilization of invading pathogens, that depend on TG-mediated cross-linking of specific proteins (6–9). Genome analysis of *Drosophila* identified a single TG gene encoding an 87-kDa protein that functions in both intracellular and extracellular spaces, although there is no authentic secretion signal sequence in the N terminus. *Drosophila* TG forms clots and traps invading pathogenic microbes (10, 11) and cross-links drosocrystallin on the peritrophic matrix, a semipermeable barrier structure in insects, to form a stabilized fiber structure against toxic proteases released by orally infected pathogenic bacteria (12). Moreover, RNAi directed against the TG gene in *Drosophila* leads to a pupal semilethal phenotype, abnormal morphology, and shorter lifespan compared with non-RNAi flies (8, 13).

Drosophila has two major immune signal pathways, the Toll and immune deficiency (IMD) pathways (14, 15). The Toll pathway is driven by yeast and Gram-positive bacteria, and the IMD pathway is driven by diamino pimelic acid-type peptidoglycans of Gram-negative bacteria or some strains of Gram-positive bacteria. The Toll and IMD pathways control the production of antimicrobial peptides by the activation of nuclear factor- κ B (NF- κ B)-like nuclear factors Dorsal/Dif and Relish (16–20), respectively. The transcriptional factor Relish, which is regulated by the IMD pathway, is endoproteolytically acti-

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³ The abbreviations used are: TG, transglutaminase; IMD, immune deficiency; NF- κ B, nuclear factor- κ B; DCA, monodansylcadaverine; CBB, Coomassie Brilliant Blue; I- κ B, inhibitor of NF- κ B; PGRP, peptidoglycan-recognition protein; rRelish, recombinant Relish; rTG, recombinant TG; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

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vated by Dredd, a homolog of mammalian caspase-8 (21). Upon stimulation with diaminopimelic acid-type peptidoglycans, Relish is converted to the N-terminal fragment (Relish-N) containing the Rel homology domain with transcriptional activity and the C-terminal fragment (Relish-C) containing an ankyrin-repeat domain that inhibits the nuclear translocation of Relish-N (Fig. 1A). We previously reported that the translocation of Relish-N from the cytosol into the nucleus is inhibited by TG-catalyzed protein-protein cross-linking in gut epithelial cells to promote immune tolerance against commensal bacteria, that full-length Relish must be proteolytically cleaved by Dredd before being cross-linked, and that the resulting Relish-N must be exposed by proteolytic conversion to become a suitable substrate for TG (13). Conversely, synthetic amines, such as monodansylcadaverine (DCA) and biotin-labeled pentylamine, ingested by flies are TG-dependently incorporated into Relish-N, which inhibits protein-protein cross-linking of Relish-N and leads to the nuclear translocation of Relish-N modified with the amines as well as native Relish-N (13). It remains unclear, however, whether the amine-incorporated Relish-N retains transcriptional activity in the nucleus. Natural polyamines, such as spermidine and spermine, are ubiquitously present in organisms and are involved in critically important intracellular functions, such as gene transcription, cellular growth, and differentiation (22–24). In the present study, we examined the effects of these polyamines on TG-dependent regulation of the transcriptional activity of a recombinant Relish-N (rRelish-N) expressed in *Escherichia coli*. Moreover, TG-dependent polyamine incorporation into native Relish-N and the effects of the modification on the transcription of antimicrobial peptide genes controlled by the IMD pathway were also examined *in vivo*.

Results

TG-dependent DCA incorporation into rRelish-N

We previously demonstrated that *Drosophila* TG catalyzes the cross-linking of Relish-N to suppress antimicrobial production (13). To confirm whether rRelish-N, as shown in Fig. 1A, functions as a substrate for TG, rRelish-N was incubated with a recombinant TG (rTG) in the presence of DCA, and the fluorescence of the DCA-incorporated rRelish was detected by UV illumination. DCA was incorporated into rRelish-N in a time-dependent manner (Fig. 1, B and C). In Coomassie Brilliant Blue (CBB)-stained gels, the amount of rRelish-N protein decreased depending on the incubation time because a part of rRelish-N was transformed into high-molecular-weight polymers through TG-dependent protein-protein cross-linking as shown in the following experiments. In addition, the incorporation of DCA into rRelish-N was equivalent to that into *N,N'*-dimethylcasein, which is a standard TG substrate, indicating that rRelish-N is a proper substrate for TG and equivalent to *N,N'*-dimethylcasein (Fig. 1D). In the present study, rRelish-N was tagged with GST derived from *Schistosoma japonicum* at its N terminus because human GST is a known substrate of TG2 (25). To confirm that the GST tag in rRelish-N is not a target of *Drosophila* TG, rRelish-N was cleaved by PreScission Protease (GE Healthcare), and the resulting proteins were incubated with

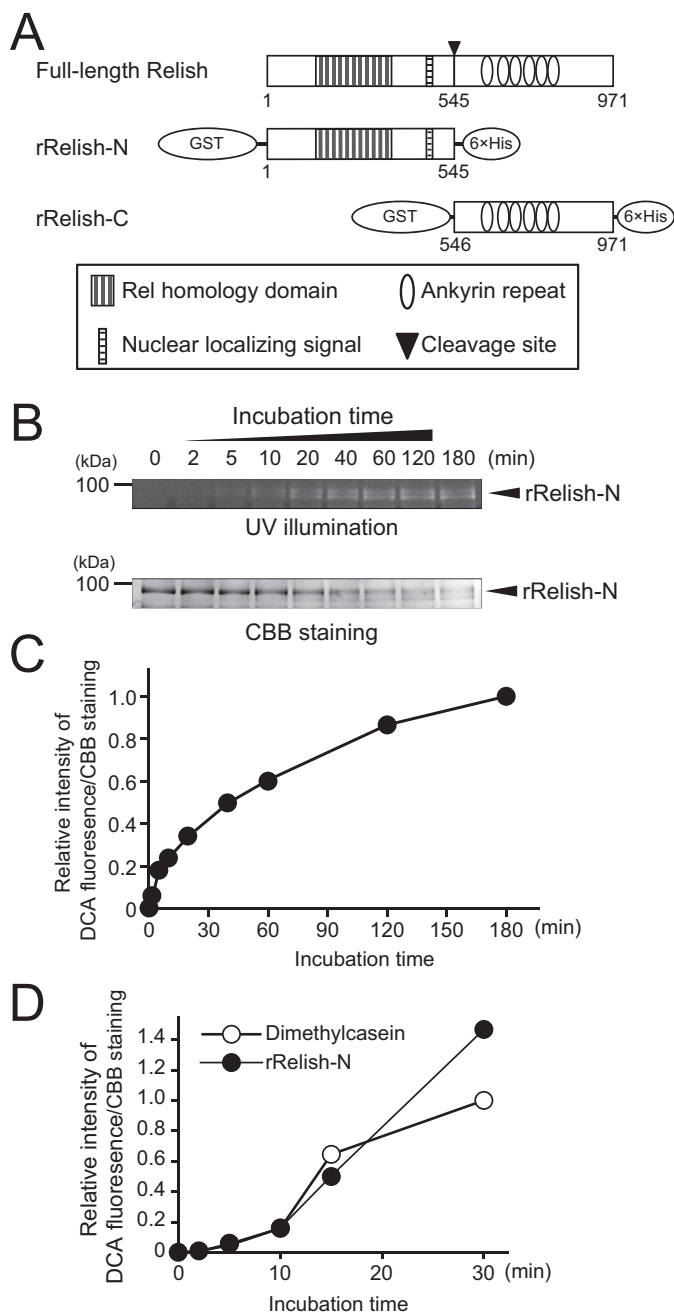


Figure 1. TG-dependent incorporation of DCA into rRelish-N. A, schematic domain structures of full-length Relish, rRelish-N, and rRelish-C. B, rRelish-N (50 nM) was incubated with rTG in the presence of DCA (1 mM) for various incubation times. Treated proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. C, density of bands stained with CBB or modified with DCA fluorescence was quantitated using ImageJ software (54), and the quantity of DCA incorporation was calculated based on CBB staining. Relative intensity of DCA fluorescence at 180 min was defined as 1.0. D, rTG was incubated with *N,N'*-dimethylcasein or rRelish-N in the presence of DCA (1 mM) for various incubation times. Treated proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. Relative intensity of DCA incorporation was quantified as described in C. Data for B, C, and D are representative of three independent experiments.

rTG in the presence of DCA. As a result, DCA was only incorporated into rRelish-N, clearly indicating that rRelish-N, but not the GST tag, is a substrate for TG (Fig. 2, A and B).

We previously reported that rRelish-N expressed in *Drosophila* S2 cells is cross-linked by TG to form a high-molecular-

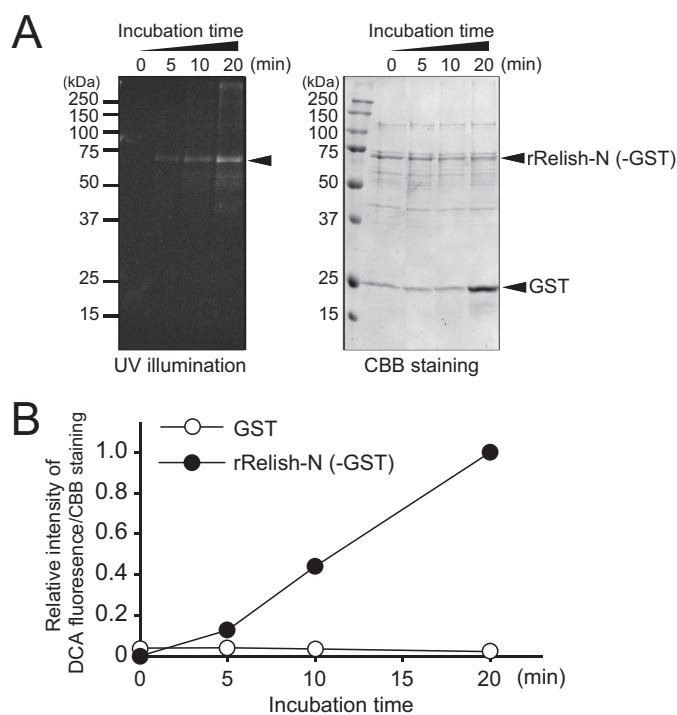


Figure 2. TG-dependent incorporation of DCA into rRelish-N without the GST tag. *A*, rRelish-N digested with PreScission Protease (2 units) was incubated with rTG in the presence of DCA (1 mM) for different incubation times. Treated proteins were separated by SDS-PAGE and detected by UV illumination (*left*) and CBB staining (*right*). *B*, CBB-stained bands and the DCA fluorescence were quantitated using ImageJ software. Data for *A* and *B* are representative of three independent experiments.

weight polymer (13). To examine whether rRelish-N can form a homopolymer *in vitro*, rRelish-N and rTG were incubated and subjected to SDS-PAGE. The high-molecular-weight bands were clearly detected by anti-His tag antibody depending on the incubation time (Fig. 3*A*, upper panel) but not by anti-TG antibody (Fig. 3*A*, lower panel), indicating that rRelish-N forms TG-dependent covalent homopolymers. We also previously reported that the full-length Relish must be proteolytically cleaved by Dredd to release Relish-N, leading to the exposure of Gln or Lys residues on Relish-N for TG-dependent cross-linking (13). In contrast, the C-terminal portion of Relish, Relish-C, contains an ankyrin-repeat domain that inhibits the dimerization of Relish-N (16–20). To examine the inhibitory effects of Relish-C on the protein-protein cross-linking of Relish-N, rRelish-N was incubated with rTG in the presence of rRelish-C. In Western blotting, the intensity of the monomer band of rRelish-N in the absence of rRelish-C was increased ~65% by the addition of an equimolar concentration of rRelish-C (Fig. 3, *B* and *C*) and increased dramatically depending on the dose increase of rRelish-C (Fig. 3*D*), indicating that rRelish-C interacts with rRelish-N through masking TG-dependent cross-linking sites on rRelish-N. To determine whether rRelish-C itself has an inhibitory effect on the cross-linking activity of rTG, TG-dependent DCA incorporation into dimethylcasein was measured in the presence of rRelish-C (Fig. 3*E*). The band intensity of DCA-incorporated dimethylcasein on SDS-PAGE was not affected by increasing concentrations of rRelish-C, indicating that the blocking mechanism of TG-dependent rRel-

ish-N polymerization is due to the presence of rRelish-C at the rRelish-N cross-linking sites.

Determination of amine-incorporation sites in Relish-N

In mammals, inhibitor of NF- κ B (I- κ B), a mammalian homolog of Relish-C, is a substrate of TG2, and the TG2-catalyzed cross-linking sites have been identified by mass spectrometry (26). To identify the amine-incorporation sites of Relish-N, rRelish-N was incubated with rTG in the presence of DCA, and DCA-incorporated Gln residues were determined by mass spectrometry after proteolytic digestion (Tables 1–3). In total, 58 peptides were identified by LC-MS/MS analysis of the tryptic or chymotryptic digestion of DCA-modified rRelish-N, covering 90% of the whole sequence of Relish-N (Tables 1–3). Six Gln residues in rRelish-N were identified as DCA incorporation sites, including Gln¹⁵⁷, Gln²¹⁹, Gln²⁷⁵, Gln²⁸⁵, Gln²⁸⁷, and Gln³⁰⁴ (Table 3). Interestingly, the modified Gln residues were all located in the Rel homology domain of Relish-N, functioning as the binding site for the κ B site of target genes (Fig. 4*A*).

To assess the mechanism underlying the specificity of the identified Gln residues for TG-dependent cross-linking of rRelish-N, all six Gln residues were mutated to Asn residues (rRelish-N^{6QN}). The expression of rRelish-N^{6QN}, however, was too low to be used for the experiments. Fortunately, rRelish-N^{5QN} with five Gln-to-Asn replacements at Gln¹⁵⁷, Gln²⁷⁵, Gln²⁸⁵, Gln²⁸⁷, and Gln³⁰⁴ was successfully prepared. rRelish-N^{5QN} was incubated with rTG in the presence of DCA and subjected to SDS-PAGE. The DCA incorporation into rRelish-N^{5QN} was strongly inhibited by the mutations, and the fluorescence intensity of the band of DCA-incorporated rRelish-N^{5QN} was ~30% that of wild-type rRelish-N (Fig. 4, *B* and *C*). Moreover, the amount of homopolymers of rRelish-N^{5QN} cross-linked by rTG was reduced to an undetectable level (Fig. 4*D*), and the ratio of Relish-N monomers to Relish-N polymers in Western blotting was significantly higher in rRelish-N^{5QN} than in rRelish-N (Fig. 4*E*), indicating a loss of the reactivity of rRelish-N^{5QN} against the TG-dependent protein-protein cross-linking. These results demonstrated that the Gln residues identified by LC-MS/MS analysis are fairly specific for TG-dependent cross-linking in rRelish-N.

Polyamine incorporation of rRelish-N inhibits its ability to bind to the κ B site

Mammalian TG2 functions not only in the cytosol but also in the nucleus (27–31). To confirm the subcellular localization of TG in *Drosophila*, rTG was expressed in S2 cells with no detectable endogenous TG antigen, and cytoplasmic and nuclear fractions were prepared. Analysis of the relative intensity of the rTG band by Western blotting indicated that the amount of rTG in the nucleus was approximately half that in the cytoplasm (Fig. 5, *A* and *B*), suggesting that *Drosophila* TG works not only in the cytosol but also in the nucleus.

DCA- or biotin-pentylamine-incorporated Relish-N is translocated into the nucleus in gut epithelial cells, but whether the amine-incorporated Relish-N retains transcriptional activity in the nucleus is unknown (13). To evaluate the DNA-binding ability of rRelish-N cross-linked with DCA, rRelish-N treated with 5 mM DCA was incubated with the biotinylated κ B oligo-

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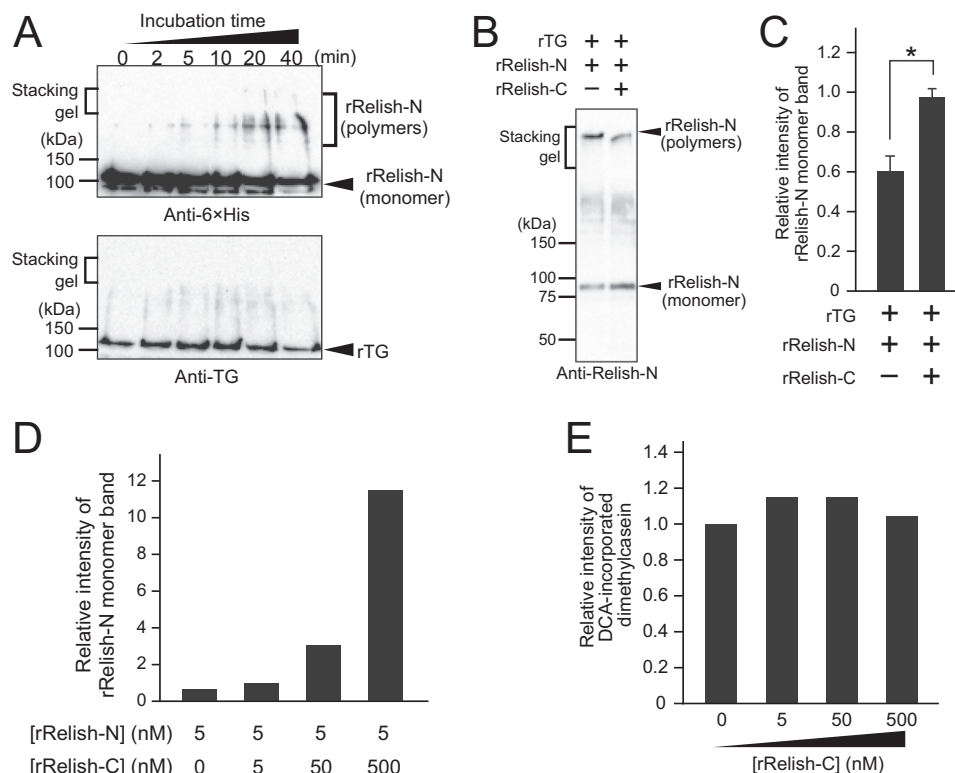


Figure 3. Polymerization of rRelish-N by TG. *A*, rRelish-N was incubated with rTG. Treated proteins were separated by SDS-PAGE and detected by Western blotting. *B*, rRelish-N (5 nM) was incubated with rTG in the presence of rRelish-C (5 nM). Treated proteins were separated by SDS-PAGE and detected by Western blotting. *C*, the intensity of rRelish-N (monomer) was analyzed by UltraQuant ID Gel Analysis Software (Aplegen). *Error bars* represent standard errors of mean values ($n = 3$). $*$, $p < 0.05$. *D*, rRelish-N (5 nM) was incubated with rTG in the presence of different concentrations of rRelish-C (0, 5, 50, and 500 nM). The intensity of the rRelish-N (monomer) band was analyzed by ImageJ software. *E*, dimethylcasein (5 nM) was incubated with rTG in the presence of different concentrations of rRelish-C (0, 5, 50, and 500 nM). The intensity of the DCA-incorporated dimethylcasein band was analyzed by ImageJ software. Data for *A*, *B*, *D*, and *E* are representative of three independent experiments.

Table 1
Mass spectrometry of trypsin-digested peptides

Mass		Δ Mass	Amino acids	Expected peptide
Measured	Calculated			
	<i>Da</i>	<i>Da</i>		
2641.098	2641.102	-0.004	14–38	K ↓ NVMFMNDASSTSGYSSSTSPNSTNR ↓ S
956.471	956.472	-0.001	39–47	R ↓ SFSPAHSPK ↓ T
748.434	748.434	0.000	148–153	K ↓ HVPQLR ↓ I
1243.692	1243.693	-0.001	154–163	R ↓ IVEQPVEKFR ↓ F
1916.878	1916.880	-0.002	166–183	R ↓ YKSEMHHGTHGSLNGANSK ↓ R
1950.948	1950.951	-0.003	188–204	K ↓ TPPEVTLCNYDGPVAVIR ↓ C
2237.097	2237.101	-0.004	205–223	R ↓ CSLFQTNLDSPHSHQLVVR ↓ K
1806.794	1806.796	-0.002	225–239	K ↓ DDRDVCDPHDLHVS ↓ E
1761.921	1761.924	-0.003	242–257	R ↓ GYVAQFINMGIHTAK ↓ K
1228.616	1228.616	-0.001	258–266	K ↓ KYIFEELCK ↓ K
1305.659	1305.661	-0.002	269–278	K ↓ QDRLVFQMNR ↓ R
1409.688	1409.690	-0.002	285–295	K ↓ QLQELHQETER ↓ E
988.476	988.476	0.000	299–306	K ↓ DMNLNQVR ↓ L
913.437	913.437	0.000	307–313	R ↓ LCFEAFK ↓ I
2537.302	2537.303	-0.001	314–336	K ↓ IEDNGAWVPLAPPVYSNAINNRK ↓ S
1228.688	1228.689	-0.001	337–347	K ↓ SAQTGELRIVR ↓ L
2112.147	2112.150	-0.003	348–367	R ↓ LSKPTGGVMGNDELILLVEK ↓ V
2319.028	2319.033	-0.005	375–393	K ↓ VRFEDEDGETVWEAYAK ↓ F
2171.009	2171.011	-0.002	396–413	R ↓ ESDVHHQYAIVCQTTPPYK ↓ D
1945.973	1945.975	-0.002	420–435	R ↓ EVNVYIELIRPSSDER ↓ S
933.507	933.507	0.000	436–443	R ↓ SFPALPFR ↓ Y
659.397	659.397	0.000	448–453	R ↓ SVIVSR ↓ K
2225.986	2225.988	-0.003	458–480	R ↓ TGSSANSSSSGTSSNNSLDLPK ↓ T
1733.835	1733.837	-0.002	508–521	R ↓ EKHLNEFIASEDFR ↓ K
1411.729	1411.731	-0.002	522–533	R ↓ KLIHNSSDLEK ↓ I

nucleotide, and the protein-oligonucleotide complex was collected using streptavidin-immobilized agarose. The amount of κ B-bound rRelish-N was significantly decreased only when incubated with rTG and DCA (Fig. 5, *C* and *D*). To confirm that

the decrease in the binding activity of DCA-treated rRelish-N to the κ B oligonucleotide was not caused by TG-dependent protein-protein cross-linking of rRelish-N, rRelish-N was incubated with rTG in different concentrations of DCA and sub-

Table 2
Mass spectrometry of chymotrypsin-digested peptides

Mass		Δ Mass	Amino acids	Expected peptide
Measured	Calculated			
	<i>Da</i>	<i>Da</i>		
1222.474	1222.463	0.011	1–9	- ↓ MNMNQYYDL ↓ D + 2 oxidation (Met)
1151.527	1151.528	-0.001	8–17	Y ↓ DLDNGKNVMF ↓ M
1031.387	1031.387	0.000	18–27	F ↓ MNDASSTSGY ↓ S
1370.606	1370.606	0.000	28–40	Y ↓ SSSTSPNSTNRSF ↓ S
1687.788	1687.788	0.000	41–55	F ↓ SPAHSPKTMELQTF ↓ A
2175.012	2175.017	-0.005	56–76	F ↓ ANLNLPGGNSPHQPPMANSFY ↓ Q
1470.727	1470.725	0.002	77–89	Y ↓ QNQLLNNGGICQL ↓ G
1279.638	1279.641	-0.002	90–102	L ↓ GATNLINSTGVSF ↓ G
1258.564	1258.565	-0.001	103–114	F ↓ GVANVTSEFGNMY ↓ M
1157.427	1157.427	0.000	111–119	F ↓ GNMVMDHQY ↓ F
1470.750	1470.751	-0.001	120–133	Y ↓ FVPAPATVPPSQNF ↓ G
787.361	787.361	0.000	134–140	F ↓ GYHQNGL ↓ A
1182.545	1182.545	-0.001	167–177	Y ↓ KSEMHGTHGSL ↓ N
1670.753	1670.755	-0.002	195–208	L ↓ CNYDGPVIRCSLF ↓ Q
1837.871	1837.874	-0.003	221–235	L ↓ VVRKDDRDVCDPHDL ↓ H
974.493	974.493	0.000	236–243	L ↓ HVSKERGY ↓ V
1387.763	1387.765	-0.001	248–259	F ↓ INMGIHTAKKY ↓ I
981.523	981.524	-0.001	282–289	L ↓ SHKQLQEL ↓ H
628.366	628.366	0.000	303–307	L ↓ NQVRL ↓ C
1767.918	1767.920	-0.002	313–328	F ↓ KIEDNGAWVPLAPPVY ↓ S
1601.812	1601.812	0.000	329–343	Y ↓ SNAINNRKSAQTGEL ↓ R
1658.854	1658.855	-0.001	349–364	L ↓ SKPTGGVMGNDELILL ↓ V + oxidation (Met)
1515.722	1515.722	0.000	392–403	Y ↓ AKFRESVDVHHQY ↓ A
1047.505	1047.506	-0.001	404–412	Y ↓ AIVCQTPPY ↓ K
1478.736	1478.737	0.000	413–424	Y ↓ KDKVDVRENVVY ↓ I
685.401	685.401	0.000	477–482	L ↓ DLPKTL ↓ G
1189.645	1189.646	0.000	483–494	L ↓ GLAQQPPNGLPNL ↓ S
1077.581	1077.582	-0.001	515–523	F ↓ IASEDFRKL ↓ I

Table 3
Dansylated peptides

Mass		Δ Mass	Amino acids	Expected peptide
Measured	Calculated			
	<i>Da</i>	<i>Da</i>		
1989.088	1989.087	0.001	148–161	K ↓ HVPQLRIVEQPVEK ↓ F + DCA (Gln)
1693.785	1693.788	-0.003	209–220	F ↓ QTNLDSPHSHQL ↓ V + DCA (Gln)
1240.607	1240.610	-0.003	272–278	R ↓ LVFQMNR ↓ R + DCA (Gln), oxidation (Met)
2045.978	2045.970	0.008	285–295	K ↓ QLQELHQETER ↓ E + 2 DCA (Gln)
1306.615	1306.616	-0.002	299–306	K ↓ DMNLNQVR ↓ L + DCA (Gln)

jected to SDS-PAGE. The polymer band of rRelish-N on SDS-PAGE disappeared at 5 mM DCA (Fig. 5E), indicating that the protein-protein cross-linking of rRelish-N by rTG was almost completely inhibited under these conditions.

Polyamines, such as putrescine, spermidine, and spermine, are natural substrates for TG (32), and intracellular polyamines are present in millimolar concentrations (33, 34). To confirm whether these polyamines are TG-dependently incorporated into rRelish-N, DCA was incubated with rRelish-N in the presence of different concentrations of spermidine or spermine. The incorporation of DCA into rRelish-N was inhibited in the presence of the polyamines in a concentration-dependent manner, suggesting that these polyamines function as substrates for TG in the cytosol or nucleus (Fig. 6, A and B). The incorporation of spermine into rRelish-N was also confirmed by Western blotting using anti-spermine antibody in the presence of DCA (Fig. 6, C and D). DCA incorporation into Relish-N was strongly inhibited by increasing concentrations of spermine, consistent with the incorporation of spermine in a concentration-dependent manner, indicating that spermine and DCA were competitively cross-linked with Relish-N by rTG (Fig. 6, E and F).

To examine polyamine modification of Relish-N *in vivo*, intestinal proteins extracted from flies fed a spermine-contain-

ing diet were analyzed by Western blotting using anti-spermine and anti-Relish-N antibodies. Protein bands of ~68 kDa corresponding to native Relish-N in wild-type flies not fed a spermine-containing diet were detected by anti-spermine antibody (Fig. 7A), and endogenous spermine was incorporated into Relish-N in the absence of exogenous spermine (Fig. 7A, left panel). The relative intensity of the ~68-kDa bands increased 2-fold depending on the amount of spermine ingested (Fig. 7, A and B). In contrast, the incorporation of spermine into the ~68-kDa bands was not detectable in *Relish*-null mutant flies (Fig. 7, A and B). These findings indicate that polyamines are incorporated into Relish-N *in vivo*. To investigate the effect of polyamine incorporation on the transcriptional activity of Relish-N, mRNA expression of the genes encoding the antimicrobial peptides cecropin A1 and dipterocin, which are controlled by the IMD pathway, was measured by quantitative PCR. S2 cells with no TG activity exhibited the same expression level of *cecropin A1* both in the presence and absence of spermine (Fig. 7C). In contrast, in cells expressing TG, the transcription of *cecropin A1* was decreased significantly in the presence of spermine (Fig. 7C). Moreover, the transcription of *dipterocin* was significantly reduced in wild-type flies fed a spermine-containing diet (Fig. 7D). In *TG*-RNAi flies, the transcriptional level of *dipterocin*

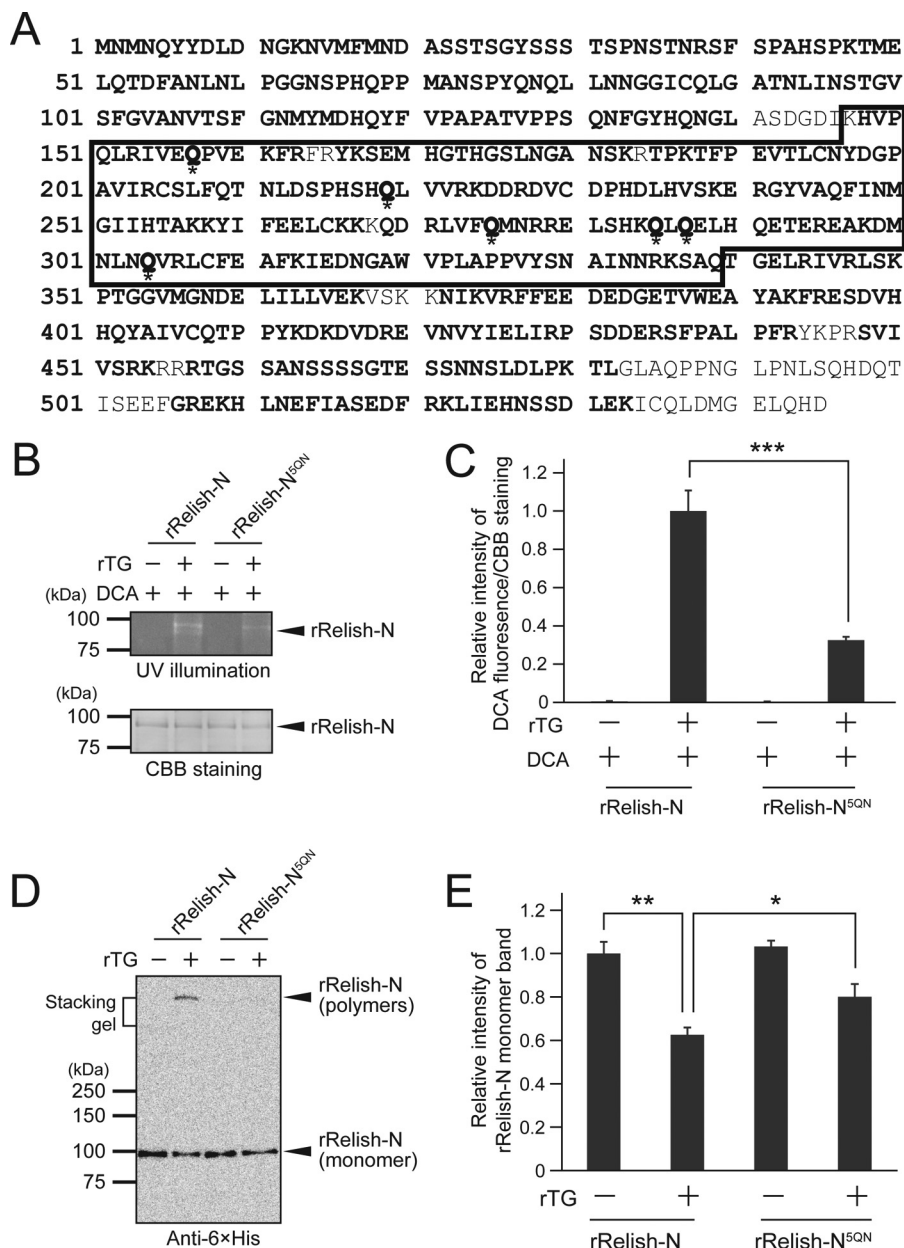


Figure 4. TG-dependent DCA incorporation into rRelish-N and rRelish-N^{5QN}. *A*, amino acids detected by mass spectrometry are shown in bold letters. Asterisks indicate the DCA-incorporated Gln residues. A box indicates the Rel homology domain. *B*, rRelish-N or rRelish-N^{5QN} was incubated with rTG in the presence of DCA. Treated proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. *C*, the bands were analyzed by ImageJ software. *D*, rRelish-N or rRelish-N^{5QN} was incubated with rTG. Treated proteins were separated by SDS-PAGE and determined by Western blotting. *E*, the intensity of the rRelish-N monomer band was analyzed by ImageJ software. Data for *B* and *D* are representative of three independent experiments. Error bars for *C* and *E* represent standard errors of mean values ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

remained constant regardless of the presence of spermine. These findings suggest that the transcriptional activity of Relish-N is controlled by polyamines in a TG-dependent manner. Fig. 7D shows that *diptericin* expression was not significantly enhanced in TG-RNAi flies because 3-day-old flies were used for the experiments. We recently found that the overexpression of antimicrobial peptide is not induced in young TG-RNAi flies (35).

Discussion

Many factors are involved in regulating the IMD pathway in *Drosophila* (36, 37): peptidoglycan-recognition protein (PGRP)-LB and PGRP-SC (amidases against peptidoglycans)

digest peptidoglycans to non-stimulatory fragments (38–40), Casper represses the expression of IMD pathway-controlled antimicrobial peptide genes (41), PIMS/Rudra/Pirk interacts with the complex of PGRP-LC (a membrane-bound receptor for peptidoglycans) and the IMD protein to reduce IMD pathway immune signaling (42–44), the Pvr receptor tyrosine kinase attenuates the JNK and NF- κ B arms of the IMD pathway (45), PGRP-LF forms a non-signaling complex with PGRP-LC (46), *Drosophila* Ring and YY1-binding protein promotes the proteasomal degradation of Relish (47), and defense repressor 1 regulates the activation of the IMD pathway in the brain (48, 49). Chronic activation of the IMD pathway leads to inflamma-

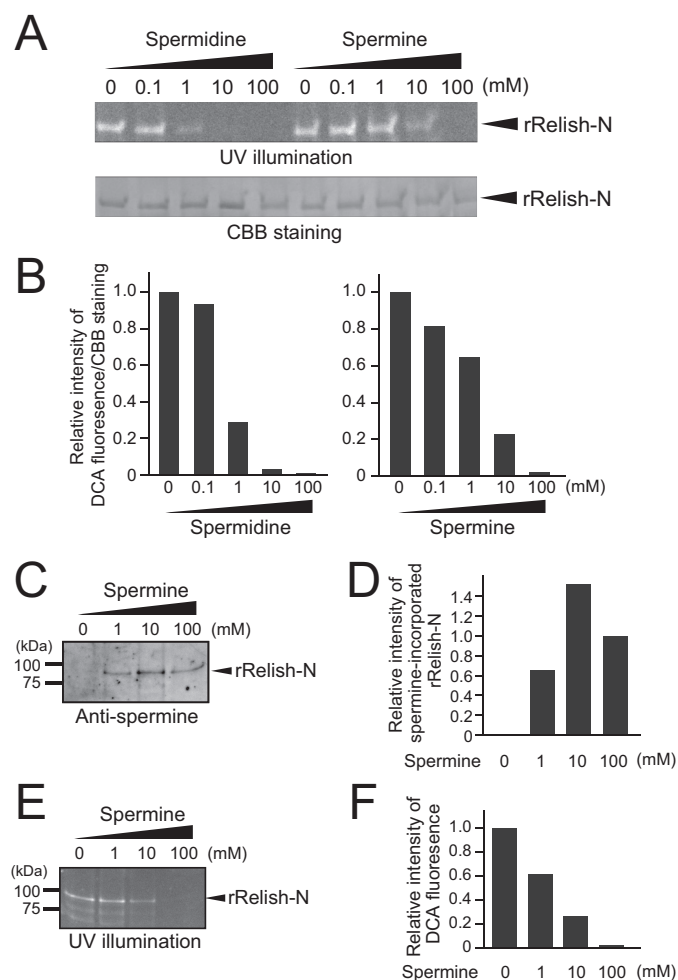
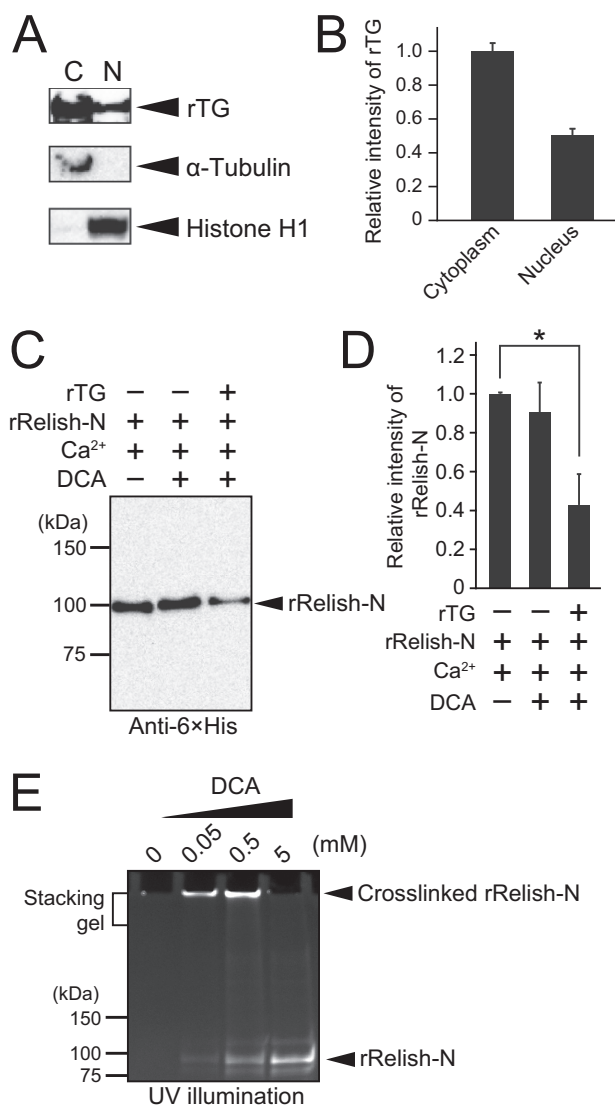


Figure 6. Polyamines and DCA were competitively incorporated into rRelish-N. A, rRelish-N was incubated with rTG in the presence of DCA (1 mM) and different concentrations of spermidine or spermine. Treated proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. B, the density of bands stained with CBB or modified with DCA fluorescence was quantitated using ImageJ software, and the quantity of DCA incorporation was calculated based on CBB staining. Relative intensity of DCA incorporation at 0 mM spermidine or spermine was defined as 1.0. C, rRelish-N was incubated with rTG in the presence of DCA (1 mM) and different concentrations of the spermine. Treated proteins were separated by SDS-PAGE and detected by Western blotting using anti-spermine antibody. D, the bands of spermine-incorporated rRelish-N were quantified by ImageJ software. Relative intensity of spermine-incorporated rRelish-N at 100 mM spermine was defined as 1.0. E, rRelish-N was incubated with rTG in the presence of DCA (1 mM) and different concentrations of spermine. Treated proteins were separated by SDS-PAGE and detected by UV illumination. F, the quantity of DCA incorporation was measured by ImageJ software. Relative intensity of DCA incorporation at 0 mM spermine was defined as 1.0. Data for A–F are representative of three independent experiments.

that the regulated amounts of antimicrobial peptides may function to maintain gut microbiota (13). In mammals, specific transcription factors involved in intracellular signaling pathways are regulated through TG2-dependent protein-protein cross-linking in the cytosol or the nucleus. Park *et al.* (26) reported an I- κ B kinase-independent activation mechanism of NF- κ B in a lipopolysaccharide-treated microglial cell line: the TG2-dependent polymer formation of I- κ B in the cytosol results in the translocation of NF- κ B into the nucleus and the subsequent up-regulation of inflammatory genes. In contrast, Tatsukawa *et al.* (30) reported hepatocyte apoptosis by TG2-

Figure 5. TG localization in S2 cells and the inhibition of the κ B-binding ability of rRelish-N by TG-dependent DCA incorporation. A, TG was expressed in S2 cells, and the cytoplasmic (C) and nuclear (N) fractions were prepared. V5-tagged rTG in the subcellular fractions was detected by anti-V5 antibody, and anti- α -tubulin and anti-histone H1 antibodies were used to detect the reference proteins of the cytoplasmic and nuclear fractions. B, the intensity of the rTG band was analyzed by ImageJ software. C, the DNA-binding ability of rRelish-N was analyzed by pull-down assay. rRelish-N was incubated with rTG in the presence of DCA (5 mM) with TG buffer at 37 °C for 1 h. The resulting DCA-incorporated rRelish-N was incubated with 50 nM biotinylated κ B oligo at 4 °C overnight. Streptavidin-immobilized agarose was added to the sample, and the resulting biotin- κ B-bound rRelish-N was obtained by centrifugation. Proteins were separated by SDS-PAGE and detected by Western blotting using anti-histidine tag antibody. D, the intensity of the Relish-N monomer band was analyzed by ImageJ software. E, rRelish-N was incubated with rTG in different concentrations of DCA (0, 0.05, 0.5, and 5 mM). Treated proteins were separated by SDS-PAGE and analyzed by UV illumination. Data for A, C, and E are representative of three independent experiments. Error bars for B and D represent standard errors of mean values ($n = 3$). *, $p < 0.05$.

tory gut diseases and reduces lifespan (50, 51), clearly indicating that distinct proteins and enzymes have evolved into essential regulatory factors to evade uncontrollable conditions for the IMD pathway.

We previously reported that TG-catalyzed Relish-N cross-linking suppresses the IMD pathway to maintain the threshold required for immune tolerance against microbes in the gut and

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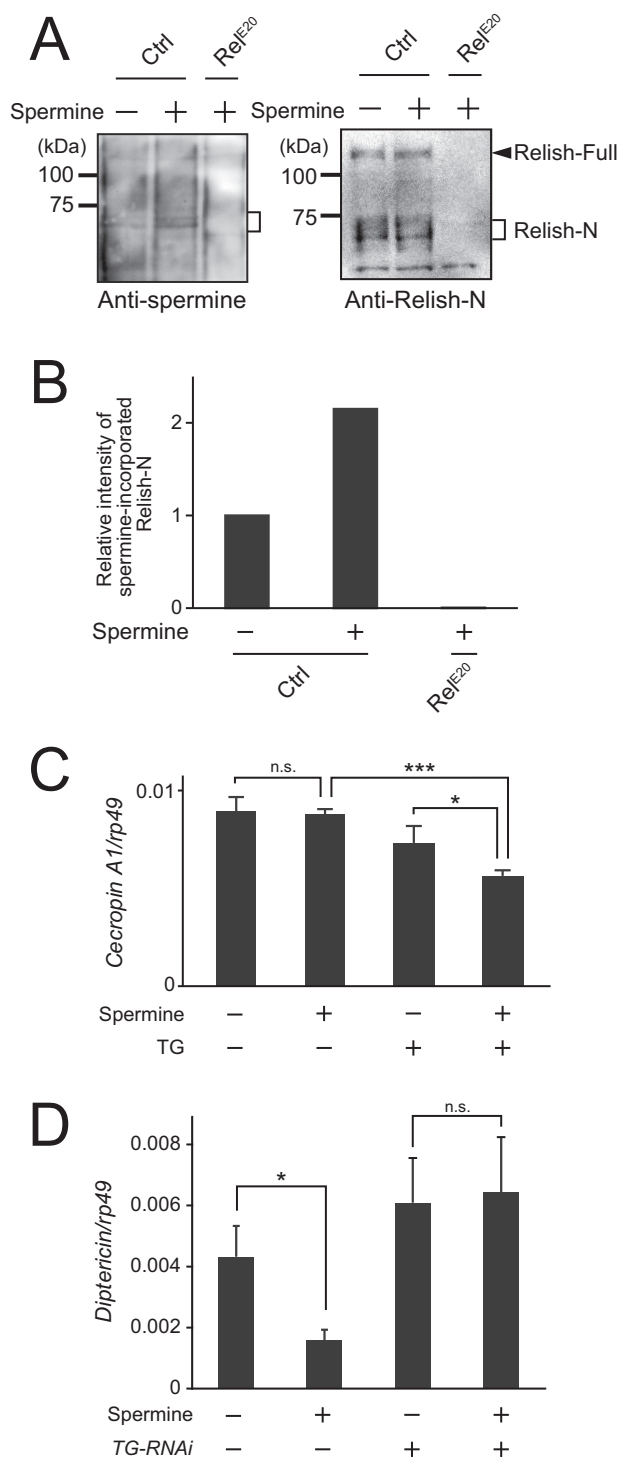


Figure 7. Polyamine incorporation into native Relish in vivo. *A*, flies were fed 5 mM spermine for 48 h and then dissected, and midguts of the flies were examined by Western blotting. *Rel^{E20}* indicates *Relish*-null mutant flies. *B*, the density of the bands of spermine-incorporated Relish-N was quantitated using ImageJ software. *C*, TG was expressed in S2 cells with medium containing 5 mM spermine. The expression level of *cecropin A1* was analyzed by quantitative PCR. *D*, *TG-RNAi* flies were fed 5 mM spermine for 48 h. The expression level of *dipterucin* was analyzed by quantitative PCR. Data for *A* and *B* are representative of three independent experiments. Error bars for *C* and *D* represent standard errors of mean values ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$; *n.s.*, not significant. *Ctrl*, control.

dependent cross-linking of the transcription factor Sp1 in the nucleus: the cross-linking of Sp1 down-regulates expression of the *c-Met* gene, which encodes a receptor for hepatocyte growth factor required for hepatocyte viability. Therefore, the regulation of signaling pathways through TG-dependent protein-protein cross-linking of specific transcription factors or regulatory components appears to be highly conserved between mammals and insects.

Conversely, our previous study revealed that synthetic amines, such as DCA and biotin-labeled pentylamine, ingested by flies are incorporated into Relish-N by TG activity, leading to nuclear translocation of the modified Relish-N into gut epithelial cells (13). Here we found that the incorporation sites for DCA identified in rRelish-N are all localized in the DNA-binding domain of Relish-N and that the DCA modification of rRelish-N reduces the binding of the κB oligonucleotide (Fig. 5, *C* and *D*). We previously reported that ingested or injected DCA triggers the activation of the IMD pathway in the fly gut, although the expression of *dipterucin* in DCA-treated flies was not as high as that in *TG-RNAi* flies (13). The present study did not investigate the transcriptional activity of rRelish-N partially modified with DCA except that DCA incorporation of rRelish-N^{5QN} was reduced to ~30% of that in wild-type rRelish-N (Fig. 4*C*). Some Relish-N partially modified with DCA that is translocated into the nucleus may retain a lower level of transcriptional activity. Natural polyamines, including spermidine and spermine, are also incorporated into Relish-N (Fig. 6). These findings suggest that the polyamine-incorporated Relish-N in the nucleus has reduced transcriptional activity in the nucleus. These polyamines are ubiquitously located in the cytosol and nucleus and play critical roles in gene regulation, cell growth, and wound healing after injury (22–24). Their intracellular concentrations are strictly regulated through metabolism and cellular import and export. We found that TG localizes not only in the cytosol but also in the nucleus in *Drosophila* S2 cells (Fig. 5, *A* and *B*). Therefore, these polyamines may be TG-dependently incorporated into Relish-N to reduce its transcriptional activity under physiologic or pathologic conditions.

TG requires Ca^{2+} for its activity (1). In addition to the production of antimicrobial peptides by the IMD pathway, reactive oxygen species-dependent innate immunity mediated by the dual-oxidase pathway in the gut is very important for host survival (52). Lee *et al.* (53) reported that uracil secreted from non-resident microbes is recognized by a G protein-coupled receptor(s), triggering the activation of phospholipase $C\beta$ to increase intracellular Ca^{2+} sufficient for spontaneous activation of the dual-oxidase pathway. We speculate that this basal concentration of Ca^{2+} in gut epithelial cells is both required and sufficient to maintain TG activity for the cross-linking of Relish-N to suppress its nuclear translocation or for polyamine incorporation into Relish to reduce its transcriptional activity (Fig. 8). We conclude that TG regulates the transcriptional activity of Relish-N through the incorporation of polyamines into Relish-N as well as through the protein-protein cross-linking of Relish-N in *Drosophila*.

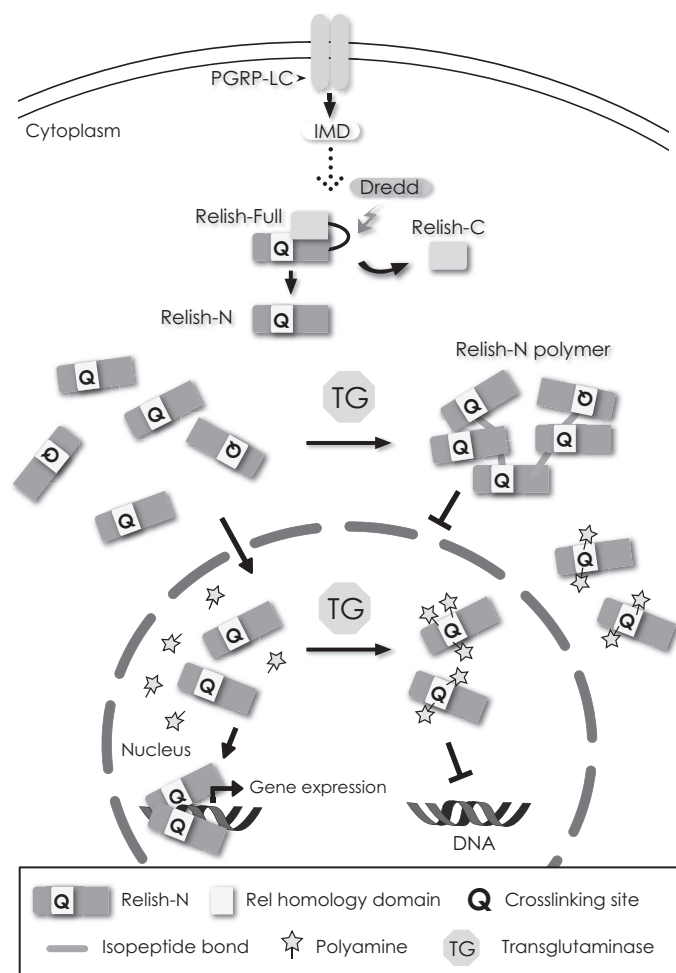


Figure 8. TG functions as a suppressor of Relish. Intracellular TG catalyzes the polymerization of Relish-N to inhibit its translocation into the nucleus. Conversely, polyamines are incorporated into Relish-N to reduce the DNA-binding or transcriptional ability of Relish-N.

Experimental procedures

Materials

All chemicals used in this study were of analytical grade unless otherwise stated. DCA, *N,N'*-dimethylcasein, and streptavidin immobilized on agarose CL-4B were purchased from Sigma-Aldrich. *Drosophila* S2 cells were purchased from Thermo Fisher Scientific (Waltham, MA). The biotinylated κ B oligonucleotide was obtained from Hokkaido System Science (Sapporo, Japan).

Fly stocks

Da-Gal4 and *w¹¹¹⁸* were obtained from Bloomington Stock Center; *UAS-TG-RNAi* was obtained from National Institute of Genetics; and *Rel^{E20}* was obtained from Prof. D. Hultmark, Umeå University. All flies were maintained in standard medium at 25 °C.

Preparation of primers for PCR

The sequence of *Relish* (CG11992) or the *TG* (CG7356) gene was amplified by PCR. For the genetic template, the complementary DNA library of *Drosophila* was used. A mutant of Relish-N was generated using a site-directed mutagenesis method.

The primers used in this study were as follows: Relish-N (residues 1–545), 5'-CGGGATCCGATGAACATGAATCAGTACTACGAC-3' (forward) and 5'-TAGTTTACGGCCGCATCGTGCTGCAACTC-3' (reverse); Relish-C (residues 546–971), 5'-CGCGGATCCGGGTCATAACCGGGCGGAA-3' (forward) and 5'-ATAGTTTACGGCCGCAGTTGGGTTAACCAGTAG-3' (reverse); TG, 5'-GGCGGATCCATGAGTTATTGGTATCGG-3' (forward) and 5'-CCGCCCCGGGAGCTATTACATCGGTGCG-3' (reverse); Relish-N (Q157N), 5'-AACCCGGTGGAGAAGTTCCGC-3' (forward) and 5'-CTCAACGATCCGCAGCTG-3' (reverse); Relish-N (Q275N), 5'-GTCTTTAACATGAACCGCCGCGAG-3' (forward) and 5'-CAGGCGATCCTGCTTCTTC-3' (reverse); Relish-N (Q285N, Q287N), 5'-CTAAACGAACTGCATCAGGAGACAGAG-3' (forward) and 5'-GTTTTTGTGGGACAACTCGCGGC-3' (reverse); and Relish-N (Q304N), 5'-AACGTGCGGCTCTGCTTTGAGGCC-3' (forward) and 5'-GTTCAAGTTCATGTCCTTGGC-3' (reverse). The constructed plasmids were confirmed by DNA sequencing.

Western blotting

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl). The blocked membrane was incubated with each antibody at room temperature for 1 h, including anti-His tag mAb-HRP-DirecT (catalogue number D291-7, MBL, Nagoya, Japan), anti-TG polyclonal antibody (8), anti-Relish-N polyclonal antibody (13), anti-V5 tag-HRP-DirecT (catalogue number PM003-7, MBL), anti- α -tubulin-HRP-DirecT (catalogue number PM054-7, MBL), anti-spermine antibody (catalogue number ab26975, Abcam, Cambridge, UK), and anti-histone H1 antibody (catalogue number 39575, Active Motif, Carlsbad, CA). Protein bands were visualized by WesternBright Quantum (Advansta, Menlo Park, CA). Chemiluminescence was detected using an Omega Lum G fluorescence imager (Aplegen, Pleasanton, CA).

Preparation of recombinant proteins

The nucleotide sequences of Relish-N and Relish-C were inserted into the pET-22b vector (Novagen, Madison, WI) containing GST and His₆ tags (Fig. 1A). For wild-type TG (rTG), the entire TG sequence was inserted into the pGEX-4T-2 vector (GE Healthcare). Transformed colonies were picked up and grown in Luria-Bertani medium to reach an optical density at 600-nm wavelength of \sim 0.6. At this stage, to induce protein expression, 0.05 mM isopropyl β -D-1-thiogalactopyranoside was added and incubated at 18 °C for 24 h with shaking at 180 rpm. After induction, the collected bacteria were sonicated in lysis buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF) by a Branson Sonifier 250 for 1 min with an output control of 2.5 and a duty cycle of 50%. The supernatant containing soluble recombinant proteins was separated by centrifugation (13,000 \times g, 4 °C, 20 min) and filtered. GST-tagged recombinant proteins were loaded on a glutathione-Sepharose 4B column (GE Healthcare). The column was washed with washing buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl)

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and eluted with elution buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 20 mM reduced glutathione).

TG-dependent DCA incorporation into rRelish-N

rRelish-N was incubated with rTG in TG buffer (50 mM Tris-HCl, pH 8.5, containing 10 mM CaCl₂, 10 mM DTT, and 1 mM DCA) at 37 °C. The resulting rRelish-N modified with DCA was precipitated with TCA and separated by SDS-PAGE. The fluorescence of DCA was detected by UV illumination (wavelength, 365 nm), and loaded proteins were also stained with CBB.

Mass spectrometry

DCA-incorporated rRelish-N separated by SDS-PAGE was detected by silver staining or CBB staining, and the protein band was excised, digested with trypsin or chymotrypsin, and subjected to LC-MS/MS analysis. Peak lists obtained from the mass spectra were used to identify fragments using the Mascot search engine (Matrix Science, Boston, MA). The analysis was carried out at the Proteomics center, Laboratory for Technical Support, Medical Institute for Bioregulation, Kyushu University.

Pulldown assay

rRelish-N was incubated with rTG in the presence of DCA with TG buffer at 37 °C for 1 h. The resulting DCA-incorporated rRelish-N was incubated with 50 nM biotinylated κ B oligo (biotin-5'-CATCGGGGATTCCTTTT-3') in the κ B-binding buffer (10 mM Tris-HCl, pH 7.5, containing 50 mM KCl, 1 mM DTT, and 1 mg/ml BSA) in the presence of 2.5% glycerol, 0.02% Nonidet P-40, and 5 mM MgCl₂ at 4 °C overnight. Streptavidin immobilized on agarose was added to the sample, and the resulting biotin- κ B-bound rRelish-N was obtained by centrifugation. The proteins were separated by SDS-PAGE and detected by Western blotting.

Subcellular fractionation of the nucleus and cytosol

Drosophila S2 cells expressing rTG were prepared as described previously (13). The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) was used to prepare the nuclear and cytoplasmic fractions according to the manufacturer's instructions.

Total RNA extraction and reverse transcription

Drosophila S2 cells were maintained at 27 °C in Insect-Xpress protein-free medium (Lonza Japan, Tokyo, Japan) with 1% penicillin-streptomycin and 50 μ g/ml gentamicin. The cells were transfected with FuGENE HD (Promega, Madison, WI). A pIB vector (Thermo Fisher Scientific) was used for expression of the C-terminal FLAG-tagged TG. Transfected cells were incubated for 18 h with 1 μ M 20-hydroxyecdysone (Sigma). After the incubation, the cells were treated with or without 0.5 mM spermine for 1 h and then stimulated with 1 μ g/ml peptidoglycan from *E. coli* O111:B4 (InvivoGen, San Diego, CA) for 8 h. The cells were washed with PBS, and total RNA was extracted with RNAiso Plus (Takara, Shiga, Japan) and treated with deoxyribonuclease I. Total RNA (500 ng) was used for reverse transcription with PrimeScript RT Master Mix (Perfect Real Time, Takara) according to the manufacturer's instruc-

tions. Three-day-old adult female flies were fed 5 mM spermine for 48 h. Spermine was dissolved in 5% sucrose. The reagent was added to a vial containing standard medium covered with glass microfiber filters GF/A (diameter, 21 mm; GE Healthcare). After feeding, flies' midguts (three replicates of three intestines) were dissected in PBS. Total RNA (300 ng) extracted from midguts was used for reverse transcription.

Quantitative PCR

Quantitative PCR was performed using FastStart Essential DNA Green Master (Roche Applied Science) on a LightCycler Nano (Roche Applied Science). Copy number was determined by the relative standard curve method. Target gene inserted into pMD19 (Takara) was used as the standard. *Ribosomal protein 49* (*rp49*) was used as a housekeeping gene. The following primers were used: *rp49*, 5'-AGATCGTGAAGAAGCGCACCAAG-3' (forward) and 5'-CACCAGGAAGTTCTTGAATCCGG-3' (reverse); *dipteracin*, 5'-GGCTTATCCGATGCCCGACG-3' (forward) and 5'-TCTGTAGGTGTAGGTGCTTCCC-3' (reverse); and *cecropin A1*, 5'-CATCTTCGTTTTCGTCGCTC-3' (forward) and 5'-CGACATTGGCGGCTTGTTGA-3' (reverse).

Spermine incorporation into rRelish-N

rRelish-N was incubated with rTG in the presence of DCA (1 mM) and different concentrations of the polyamines. Treated proteins were separated by SDS-PAGE and detected by UV illumination and Western blotting with anti-spermine antibody. To detect spermine incorporated into Relish, flies were fed 5 mM spermine for 48 h. Spermine-fed flies were dissected, and midgut proteins were extracted by lysis buffer. The proteins were separated by SDS-PAGE and confirmed by Western blotting.

Statistical analysis

Student's *t* test was used to calculate *p* values. A *p* value of less than 0.05 was considered significant.

Author contributions—K. M. and T. S. performed the experiments. K. M., T. S., and S. K. analyzed the data and interpreted the experimental results. K. M., T. S., and S. K. designed the experiments and wrote the paper.

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