J. Biochem. 127, 1127–1134 (2000)

Molecular Cloning and Characterization of SRAM, a Novel Insect Rel/Ankyrin-Family Protein Present in Nuclei[1]

Hirohisa Shiraishi,* Ayako Kobayashi,† Yasumitsu Sakamoto,‡ Takamasa Nonaka,‡ Yukio Mitsui,‡ Naohiko Aozasa,* Takeo Kubo,* and Shunji Natori[1,2]

**Department of Cell Biochemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033; †Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871; ‡Department of BioEngineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2137; and §The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198*

Received February 2, 2000; accepted April 10, 2000

Previously, we purified a 59-kDa protein that binds to the κB motif of the *Sarcophaga* lectin gene. Here we report its cDNA cloning and some of its characteristics as a novel member of the Rel/Ankyrin-family. This protein, named SRAM, contained a Rel homology domain, a nuclear localization signal and 4 ankyrin repeats, but lacked the Ser-rich domain and PEST sequence that Relish contained. We found that SRAM was localized in the nuclei of NIH-Sape-4 cells, which are an embryonic cell line of *Sarcophaga*. The *Sarcophaga* lectin gene promoter containing tandem repeats of the κB motifs was activated in NIH-Sape-4 cells. In *Drosophila* mbn-2 cells, Dif alone activated this reporter gene and a cooperative effect was detected when SRAM and Dif were co-transfected, although SRAM alone did not activate it. This is the first report of a Rel/Ankyrin molecule that exists in the nuclei.

Key words: ankyrin, insect immunity, κB motif, NF-κB/Rel family, *Sarcophaga peregrina*.

Insects have developed self-defense systems that recognize pathogens and destroy them through cellular reactions, and that produce various antimicrobial peptides (*1–3*). This defense system sans antibody, called insect immunity, has been investigated vigorously in several insect species. Recent studies have revealed that there is a common molecular basis between insect immunity and the innate immunity of vertebrates (*4, 5*).

Transcription factors of the NF-κB/Rel family are well-investigated immune regulatory molecules involved in mammalian as well as insect self-defense systems (*6–11*). All NF-κB/Rel proteins have a Rel Homology Domain (RHD), which is required for DNA-binding and dimerization of two subunits. Translocation from the cytoplasm to the nucleus is a critical step needed for NF-κB to express its transcription factor activity in response to various immune stimuli. NF-κB is arrested in the cytoplasm by IκB, a family of NF-κB inhibitors possessing ankyrin repeats. Immune stimuli induce the dissociation of NF-κB/IκB complex and degradation of IκB, resulting in migration of NF-κB into the nucleus (*6–8*). Precursors of NF-κB such as p105 and p100 contain both RHD and ankyrin repeats like IκB, but the ankyrin repeats are processed during maturation. The resulting p50 or p52 forms homodimers as well as heterodimers with other Rel family proteins and finally with IκB to produce an NF-κB/IκB complex (*12*).

In insects, four Rel-related proteins (Dorsal, Dif and Relish in *Drosophila melanogaster*, and Gambif-1 in *Anopheles gambiae*) have been cloned (*2, 13–16*). Dorsal, Dif and Gambif-1 contain both a RHD and a transactivation domain, and are therefore assumed to be p65 (mammalian RelA) homologues of insects. In contrast, Relish, which contains a RHD and an inhibitory ankyrin domain, is thought to be a p105 or p100 homologue (*15*). Recently, Han and Ip showed that the expression of a battery of *Drosophila* antibacterial and anti-fungal protein genes is regulated differentially with distinct heterodimeric or homodimeric combinations of Dif, Dorsal, and Relish (*17*). Cactus, an IκB homologue in *Drosophila*, is also able to regulate these *Drosophila* Rel proteins (*18*).

We have been studying the mechanism of activation of various immune protein genes in the flesh fly, *Sarcophaga peregrina* (*19–23*). Previously, we reported the purification and characterization of a 59-kDa protein (*24*) that binds specifically to the κB motifs in the 5′ upstream regions of these immune protein genes. In this study, we report that this 59-kDa protein named SRAM (*Sarcophaga*-derived Rel/Ankyrin Molecule) is a novel member of the Rel/Ankyrin-family proteins. We found that SRAM, in contrast to known Rel family proteins, was localized in the nucleus irrespective of immune stimuli. SRAM by itself did not activate the promoter of the *Sarcophaga* lectin gene in a *Drosophila* cell line, but it enhanced the activity of co-transfected

[1] This work was supported by a Grant-in-Aid for Science Research from the Ministry of Education, Science, Sports and Culture of Japan (No.11147207, No.1160205) and by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST).

[2] To whom correspondence should be addressed. Phone: +81-48-467-9437, Fax: +81-48-462-4693, E-mail: natori@postman.riken.go.jp

Abbreviations: FBS, fetal bovine serum; LPS, lipopolysaccharide; RACE, rapid amplification of cDNA ends.

Dif. It could be a nuclear regulatory molecule that functions cooperatively with Rel proteins or unknown co-factors to modulate the tissue-specific and/or inducer-specific expression of the immune genes.

MATERIALS AND METHODS

Cells and Culture Media for Purification of SRAM—The embryonic cell line of *S. peregrina*, NIH-Sape-4, was cultured in M-M medium at 25°C as described by Komano *et al.* (*25*). Briefly, NIH-Sape-4 cells were inoculated at a density of about 5×10^5 cells/ml in 4 liters of M-M medium in a spinner flask and cultured with constant stirring and aeration for 7 days. Cells were harvested when the cell density reached about 4×10^6 cells/ml.

Purification of SRAM and Determination of Its Partial Amino-Acid Sequences—SRAM was purified from NIH-Sape-4, as described by Kobayashi *et al.* (*24*). Proteolytic fragments of SRAM were obtained as follows. The purified SRAM (33 μg) was subjected to SDS–polyacrylamide gel electrophoresis, and blotted onto a poly (vinylidene difluoride) filter paper. After staining the filter paper with Ponceau S, the band corresponding to the SRAM was cut off. This filter strip was destained with 0.2 mM NaOH for 1 min, and then incubated for 30 min at 37°C with 0.5% PVP-40 in 100 mM acetic acid. The filter strip was then cut into small pieces (1 × 1 mm) and treated with 12 μg/ml lysyl-C endopeptidase. The resulting peptides were released from the filter pieces by two 30 min sonications and applied to an HPLC system with a reverse-phase column of Synchropak RP-P (C_{18}). The fractions containing each peptide were lyophilized and subjected to automated sequence analysis. By this procedure, the sequences of 10 peptides were determined. These were: peptide 1, IVLSPHLLQHN-(T/G)EK; peptide 2, LNYDVIFERMQEDPK; peptide 3, KI-HPETMETEWERFVNVK; peptide 4, DIFGNHAITFITPR-YD; peptide 5, PYSVVDNEPSTDNXRN; peptide 6, N(D/M)(M/D)VAKGEETVLDNSAIFN; peptide 7, ETNPQIFK; peptide 8, YLQNLSQYPESVK; peptide 9, KIELFENLLK; and peptide 10, DYLLDK.

Cloning Procedure and Sequencing of cDNA—Two primers corresponding to IHPETMET and NHAITFIT in peptides 3 and 4, respectively, were synthesized as 5′-ATCCA-CCCAGA(AG)AC(AGCT)ATGGA(AG)AC-3′ and 5′-GTGAT-GAAGGTAAT(AGCT)GC(AG)TG(AG)TT-3′. The PCR reactions were performed with 0.5–1 nmol of each primer, 5 μg of template DNA (cDNA library of NIH-Sape-4 cells) and AmpliTaqGold (Perkin Elmer) in 100 μl of standard reaction mixture. After activation of the polymerase for 9 min at 95°C, we repeated the following 40 cycles (1min at 95°C, 1min at 40°C, 2 min at 72°C) with a final 5 min extension at 72°C. A major band of about 100 bp was amplified. We sequenced this product by a direct sequencing method and found that it was a 92 bp DNA fragment derived from SRAM cDNA. This fragment was used as a probe to screen SRAM cDNA. To screen SRAM cDNA efficiently, a cDNA library of NIH-Sape-4 cells containing ~10^5 independent clones was divided into sub-libraries, each library containing ~10^3 independent clones. About 24,000 colonies of *Escherichia coli* SOLR™ strain carrying recombinant pBluescript (Stratagene), which were derived from 4 PCR-positive sub-libraries, were transferred to duplicate sets of nylon filters. The hybridization procedure was essentially the same as that described by Nakanishi-Matsui *et al.* (*23*). One hybridization positive clone with the probe DNA was obtained and sequenced by the dideoxy chain termination method of Sanger *et al.* (*26*) using a Taq Primer Cycle Sequencing Kit (Applied Biosystems). This clone contained a 3′-region of SRAM cDNA (about 1.3 kbp) and lacked a 5′-region. This missing 5′-region was isolated by a 5′-RACE method with CLONTECH Marathon™ cDNA amplification kit, cloned with a pGEM™-T vector system I (Promega), and sequenced. In this way we determined the complete sequence of SRAM cDNA.

Preparation of Affinity-Purified Antibody against SRAM—Antibody against SRAM was raised by injecting approximately 10 μg of the purified protein with complete Freund's adjuvant into a male albino rabbit (Japanese white) and then giving 3 booster injections of the same amount of protein with incomplete Freund's adjuvant every week. We performed affinity purification of the resulting antibody with purified SRAM, essentially as described before (*27*). For this, purified SRAM fraction (10 μg) was first electrophoresed on a 10% polyacrylamide gel containing 0.2% SDS, and then the proteins were blotted onto a poly (vinylidene difluoride) filter paper. The small region of the filter, on which SRAM had been concentrated, was excised and treated with 5% skim milk solution. The strip of filter paper was then incubated in 1 ml of 2- to 3-fold–diluted antiserum at 4°C for 15 h with gentle shaking. The strip was rinsed well with rinse solution (10 mM Tris/HCl pH 7.9, containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, 0.01% sodium azide and 0.25% skim milk), and the antibody specifically bound to SRAM was extracted with 0.2 M glycine/HCl (pH 2.8). The resulting extract was neutralized with 1 M KOH, and a final concentration of 10% bovine serum albumin was added.

For the preparation of the antibody against the ankyrin domain of SRAM, the C-terminal half fragment of SRAM was obtained by proteolytic digestion of recombinant SRAM. Recombinant SRAM (50 μg) was incubated with 0.2 μg of Trypsin in 50 μl of 10 mM Tris/HCl (pH 8.0) at 20°C for 1 h and then the resulting fragment with the molecular mass of 24 kDa was used as the antigen for affinity-purification of the anti–SRAM-ANK antibody. The N-terminal sequence of this proteolytic fragment starts at the 353th amino acid (Val).

Preparation of Recombinant SRAM—The cDNA encoding full-length SRAM (586 amino-acid residues) was cloned into the His-Tag expression vector pET23d (Novagen). The fusion protein, with His_6 at its C-terminal, was expressed in *E. coli* BL21(DE3) using the pET expression system, and then purified to homogeneity with His·Bind Resin (Novagen).

Electrophoresis Mobility Shift Assay—Binding reactions were performed in the binding buffer [12 mM HEPES-NaOH (pH 7.6), 12% glycerol, 0.1 mM EDTA, 60 mM KCl, 7.5 mM $MgCl_2$, 0.3 mM dithiothreitol], containing 20 fmol of ^{32}P-5′-end–labeled double-stranded DNA (catGGGAA-TACCCtg) and various concentrations of purified recombinant SRAM. When necessary, 300-fold excesses of specific (catGGGAATACCCtg) or non-specific (catATTAACAAG-Gtg) competitors were added. The reaction mixture was kept on ice for 30 min and then analyzed by electrophoresis with a non-denaturing 5% polyacrylamide slab gel at 4°C in a solution of 45 mM Trisma base, 45 mM boric acid, and 1

mM EDTA. The gel was then dried and examined by autoradiography.

Indirect Immunofluorescence Staining—NIH-Sape-4 cells were cultured for two days in the Grace's insect medium (pH 6.0) (GIBCO), containing 5% fetal bovine serum (FBS). Then the cells were suspended in 5% FBS/Grace's insect medium at a density of approximately 10^6 cells/ml, and 20 μl of the suspension was placed in each well of a 12-well multitest slide. The cells were treated with 100 μg/ml of LPS from *E. coli* O55:B5 for 2 h when necessary, and then fixed for 10 min by soaking the slide in 4% paraformaldehyde/0.02% glutaraldehyde/phosphate-buffered saline. The slides were rinsed with phosphate-buffered saline and blocked with 100 mM glycine/1% bovine serum albumin/phosphate-buffered saline for 1 h at room temperature. Then 15 μl of affinity-purified antibody solution (5 μg/ml) was added to each well, and the slides were incubated for 2 h at room temperature. The slides were then treated with FITC-labeled secondary antibody for 1 h at room temperature, washed, mounted, and examined with an Olympus BH-2 fluorescence microscope.

Transfection Experiment—Drosophila mbn-2 cells were cultured for 3 days at 25°C in Schneider's *Drosophila* medium (GIBCO) supplemented with 12% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was then diluted with fresh medium to adjust the cell density to 4×10^5 cells/ml. One millilitter of the resulting cell suspension was transferred to each well (3.5 cm diameter) of the 6 well-plate and the culture was continued for 24 h. The culture medium was then replaced with 1 ml of antibiotic-free *Drosophila* Serum Free Medium (GIBCO), and the cells were transfected by the CELLFectin (GIBCO) lipofection method. For this, 1 μg of the expression vector [SRAM, dorsal, or Dif cDNA in the pPAC-pl vector (*28*)], 1 μg of the reporter vector [(κB)3-*Sarcophaga* lectin promoter-luciferase] and 0.2 μg of the β-galactosidase expression vector (pACH110) (*29*) as an internal control for the transfection efficiencies, were mixed in 200 μl of *Drosophila* Serum Free Medium containing 8 μl of CELLFectin. The total amount of pPAC vectors was adjusted to 2 μg with the pPAC-pl vector. This DNA solution was kept for 30 min at room temperature, and then mixed with an additional 800 μl of *Drosophila* Serum-Free Medium. This mixture was added directly to each well and incubated for 4 h. Then this transfection solution was removed, and 2 ml of Schneider's complete medium was added. The gene activities were assayed 48 h later. The cells from each well were collected and washed, and then lysed in the reporter lysis buffer (Promega) for 10 min. Luciferase activity in the lysate was measured in a luminometer (Lumat LB 9507; BERTHOLD) immediately after addition of the substrate Luciferin (Promega), according to the protocol of the distributor. To normalize transfection efficiency, β-galactosidase activity in the lysate was also measured, using *O*-nitro-phenol-β-D-galactoside as a substrate.

RESULTS

cDNA Cloning of SRAM—We isolated a cDNA clone for SRAM. The longest open reading frame of this cDNA initiated from an AUG codon at nucleotide position 66 and encoded a protein consisting of 586 amino-acid residues (Fig. 1A). All of the 10 peptides that were derived from purified SRAM were included in this sequence, suggesting that this putative protein was SRAM. However, the molecular mass of this protein was calculated to be 67.8 kDa, whereas that of purified SRAM determined by SDS polyacrylamide gel electrophoresis is 59 kDa, as reported previously (*24*).

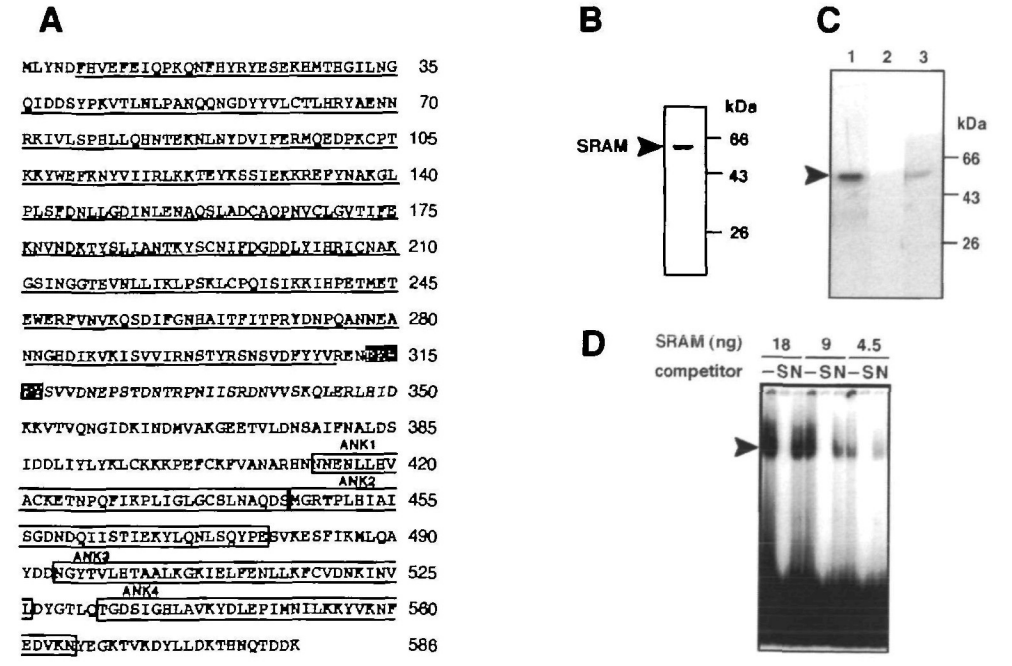
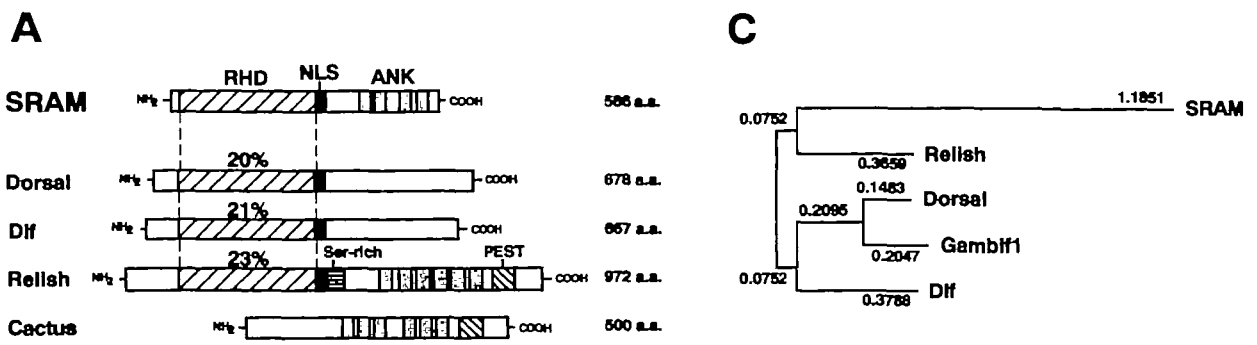


Fig. 1. **Primary structure of SRAM and production of recombinant.** (A) Predicted amino-acid sequence encoded by the SRAM cDNA. The Rel homology domain is underlined and the ankyrin repeats (ANK1–4) are boxed. A putative nuclear localization signal is shown in white on a black ground. The nucleotide sequence data for SRAM has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB035929. (B) SDS–polyacrylamide gel electrophoretic profile of purified recombinant SRAM. The gel was calibrated with bovine serum albumin (66 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (26 kDa). (C) Immunoblot analysis of recombinant SRAM. Lane 1, purified native SRAM (0.4 μg); lanes 2, 3, lysates of *E. coli* (2.5 μg) without or with SRAM expression vector, respectively. (D) Electrophoresis mobility shift assay with various amounts of purified recombinant SRAM and the κB motif of the *Sarcophaga* lectin gene. Competitor-, S and N indicate addition of no competitor, and a 300-fold excess of specific and non-specific DNA, respectively. The arrowhead indicates the specific DNA-SRAM complex.

Therefore, we expressed this cDNA in *Escherichia coli* and synthesized a recombinant SRAM. The molecular mass of purified recombinant SRAM examined by SDS polyacrylamide gel electrophoresis was 59 kDa (Fig. 1B). Moreover, the recombinant SRAM was recognized by antibody against purified SRAM (Fig. 1C), and showed a specific binding activity to κB element in the *Sarcophaga* lectin gene (Fig. 1D). These results indicate that this cDNA encoded SRAM.

SRAM as a Rel/Ankyrin-Family Protein—The deduced amino-acid sequence of SRAM contained both a Rel homology domain (RHD) in the N-terminal half and ankyrin repeats in the C-terminal half. A putative nuclear localization signal (NLS) was found at the C-terminal end of the RHD (Figs. 1A and 2A). In these respects, SRAM was similar to the mammalian NF-κB precursors, p100 (a precursor of p52), p105 (a precursor of p50), and a *Drosophila* Rel family protein, Relish. However, there are several differences between known Rel/Ankyrin-family proteins and SRAM (Figs. 1A and 2A). First, known Rel/Ankyrin-family proteins contain 6 or more ankyrin repeats, but SRAM has only 4. Second, whereas p100 and p105 have a Gly-rich region that serves as a processing signal for the generation of p52 and p50, such a characteristic region is not present in SRAM. SRAM does not possess even a Ser-rich stretch, which Relish has at the position corresponding to a Gly-rich region of p100 or p105 (*30*). The sequence of 95 residues between the NLS and the first ankyrin domain of SRAM is unique, and is not like those of the same regions of other Rel-family proteins. Several Ser residues were found in this region, but the consensus phosphorylation motif, (D/E/N)XXXDSGXXS, in the N-terminal region of inhibitors of NF-κB (IκB-α, IκB-β, IκB-ε, and Cactus) for a phosphorylation-induced proteolytic degradation in extracellular stimuli, was not identified (*6*). Third, SRAM has a short C-terminal region containing only 21 residues behind the last ankyrin domain, but there was no PEST-like sequence (*31*) like those existing in p100, p105, and Relish for protein turnover. These structural features suggested that



B

```
SRAM        6 FHVEFEIQ-KCNFHYF-ES-KHMTHCILNGQIDDSYP-VTLNLPANQQN-DYYVLCTL--HRYAENN-RKIVLSPHLL  80
Relish    150 PQLRIVEQ-VEKFRF-KS-MHGTHGSLNGANSKRTP-TFPEVTLCNYD-PAVIRCSL----F-QTN-LDSPHSHQLV 221
Dorsal     47 PYVKITEQ-AGKALRF-EC-GRSAGSIPG-VNSTPE-NYTYPIEIVGYK-RAVVVVSCVTKDTPYRPHPHNLVGK---E 122
Dif        78 PHLRIVEE-TSNIIRF-KC-GRTAGSIPG-MNSSSETG-TFPTIEVCNYD-PVIIVVSCVTSDEPFRQHPHWLVSK--EE 155
RelA(P65)  19 PYVEIIEQ-KQRGMRF-KC-GRSAGSFTIPGERSTDTT-THPTIKINGYT-PGTVRISLVTKDPPHRPHPHELVGKDCRD  99
p105(p50)  43 PYLQILEQ-KQRGFRF-VC-GPSHGG--LPGASSEKNK-SYPCVKICNYV-PAKVIVQLVTNGKNIHLHAHSLVGKHCED 121

SRAM       81 QHNTEKNLNYDVIFERMQEDPKCPTKKY-WEFKNYVIIR-LKKTEYKSSIEKKRE--F-YNAKGLPLSFDNLLG-DINLEN 155
Relish    222 VRKLDRDV-CDF-HD-LH-VSK--ERGYVAQFINMGIIHTAKKYIFEELCKKKQDR-LVFQMNRRELSHKQLQELHQETER 295
Dorsal    123 ----GC---K--KGVCTLEINSE-TMRAVFSNLGIQ----C----VKK-KDIEAAL-KAREEIRVDPFKTGFSH-RFQPSS 182
Dif       156 A--CAC---K--SGIYQKKIPPE-ERRLVLQKVGIQ----C----AKK-LEMRDSL-VERERRNIDPFNAKFDH-KDQIDK 217
RelA(P65) 100 GFY--EA---EIFTC-PDRCI-H-S----FQNLGIQ----CVK--KRD-LEQA-IS-QRIQ-TNNNPFQVPIEE-QR-GDY 156
p105(p50) 122 GICTVTAGPKDPVVGFANLCILHVTKKKVFETLEARMTEACIRGYNPGLLVHPDLAYLQAEGGGDRQLGDREKELIRQAAL 202

SRAM      156 AQSIADCAQ-PNVCL-GVTIFEKNV-NLKTYSLIANTKYSCNIFDGDDPYTHG-ICNAKGSINSGTEVNLPIKLPSPLCPQ 232
Relish    296 EAKCMNLNQ-VRLCFEAFKIEDNGAWVPLAPPVYSNAINNRKSAQTGERRIVP-LSKPTGGVMPNDEIILIVEKVSPKNIK 374
Dorsal    183 IDLNSVRLC-FQ-VF-MESEQ-KGRFTSPLPPVVSEPIFDKK--AMSDPVICPLCSCSAI-VFPNTQIILICEKVAPEDIS 256
Dif       218 INRYELRLC-YQ-AF-ITVG--NSK-V-PLDPIVSSPIYG-K--S-SEPTPTPLCSCAAI-ANPGDEIIMLCEKIAPDDIE 286
RelA(P65) 157 -DLNAVRLC-FQ-VFTT-VRDPSGR-PIRLPPVLPHPIFDNRAPNTAELKECPVNRNSGSCL-PGDEIFLLCDKVQPEFTD 231
p105(p50) 203 QQTTEMDLSVVRLMFTAFLFDSTGSFTRRLEPVVSDAIYDSKAPNASNLKIVSMDRTAG-CVTPGEEIYLLCDKVQKDDIQ 282

SRAM      233 ISIKKIHPETMETELERFVNVKQSPIFGNHPLTFIHRRDNPQANNEANNGHDIKVKISVVIRNSTYRSNSVDPYPVR  310
Relish    375 VRFFE-EDEDGETVPEAYAKFRESPVHHQYPLVCQPPPKDKDVDREV-NVY-IEL-IRFSDDERSF-P-ALPPRKP  446
Dorsal    257 VRFFE--EKNGQSVPEAFGDFQHTPVHKQTPLTFKPPRR----HTLDITEPAKVFIQLRRPSD-G-VTSEALPPEVP  326
Dif       287 VRFYE-TDKDGRETPFANAEFQPTPVFKQMPLAFKPPRR----RNTEITQSVNVELKLVRPSD-G-ATSAPLPPEYP  357
RelA(P65) 232 IEVY----FTGFG-PEARGSFSQALVHRQVPLVFRQPPP----ADPSLQAPVRVSMQLRRPSDRELSFTEPMEPQLP  300
p105(p50) 283 IRFYE--EEENGGVPEGFGDFSPTPVFRQPPLVFKPPKH----KDINITKPASVFVQLRRKSD--LETSEPKPPTLYP  352
```

Fig. 2. **Comparison of SRAM with other Rel/Ankyrin-family proteins.** (A) Schematic illustration of SRAM and other Rel/Ankyrin-family proteins of *Drosophila* with amino-acid sequence identity among the RHD. (B) Comparison of amino-acid sequence of the RHD of SRAM with those of other Rel family proteins. Identical residues are shown in white on a black ground, and relatively conserved residues (more than 50%) are shown in black on a gray ground. Amino-acid residues essential for DNA binding and dimerization of NF-κB (p65/p50) are underlined in red and blue, respectively. An asterisk indicates the Cys residue involved in redox regulation of NF-κB. (C) Phylogenetic analysis of SRAM using the UPGMA method. The numbers in the tree denote genetic distances.

SRAM was not a *Sarcophaga* homologue of p100, p105, or Relish, but a novel member of the Rel/Ankyrin-family.

We compared the amino-acid sequence of the RHD of SRAM with those of known members of the Rel family in insects and vertebrates (Fig. 2B). Three subdomains were conserved relatively well. These were one N-terminal subdomain of the RHD (residues 13 to 25), and two C-terminal subdomains (residues 201 to 228 and 247 to 271). The former contains the essential amino acid residues for DNA binding and the others are important for dimerization (*32–36*). However, the other regions were different, except for a few well-conserved residues. In the N-terminal DNA-binding subdomains of RHD, a Cys residue, indicated by an asterisk, is well conserved among Rel-family proteins. This Cys is known as a target for thioredoxin, a redox regulator of the DNA-binding activity of NF-κB (*37, 38*). It is noteworthy that this Cys is missing in both SRAM and Relish. A phylogenetic comparison of the RHD of various Rel-family proteins suggested that SRAM and Relish belonged to the same subfamily, and that this subfamily had branched off from the other Rel family early in the evolution of Rel-family proteins (Fig. 2C). However, amino acid sequence identity between RHD of SRAM and that of Relish was only 23%.

Nuclear Localization of SRAM—The activity of the Rel family transcription factors is regulated at the level of nuclear translocation in response to microbial infection and oxidative stress (*39*). NF-κB is normally localized in the cytoplasm as an inactive form of NF-κB/IκB complex, but translocated into the nucleus with dissociation and degradation of IκB (*6–8*). As reported previously, we purified SRAM from a nuclear extract of NIH-Sape-4 cells. These cells were shown to synthesize constantly several immune proteins including *Sarcophaga* lectin (*24*), when cultured in M-M medium, because this medium contains yeast extract. These immune protein genes are inactive when the cells are cultured in modified Grace's insect medium, but the genes are activated when microbial cell wall components such as LPS or β-1,3-glucan is added to the medium.

We performed immunofluorescence staining with affinity-purified antibody against SRAM using NIH-Sape-4 cells cultured in Grace's insect medium in the presence or absence of LPS. Immunofluorescence was detected mainly in the nuclei, irrespective of the presence of LPS (Fig. 3A). This distribution of SRAM was quite different from those of other Rel-family proteins (*6–8*). We obtained the same result using antibody affinity-purified with the C-terminal half of recombinant SRAM obtained by cleaving it between NLS and the first ankyrin domain with trypsin (Fig. 3, B, C, and D). These results indicated that much of SRAM was localized in the nuclei, and confirmed the result of our immunoblotting experiments with NIH-Sape-4 cells (data not shown). Therefore, it is likely that, at least in the NIH-Sape-4 cells, activation of immune protein genes does not require migration of SRAM from the cytoplasm to the nuclei. Similarly, we detected an intense signal of SRAM in the nuclei of fat body cells of *Sarcophaga* larvae, regardless of SRBC injection, which causes activation of various im-

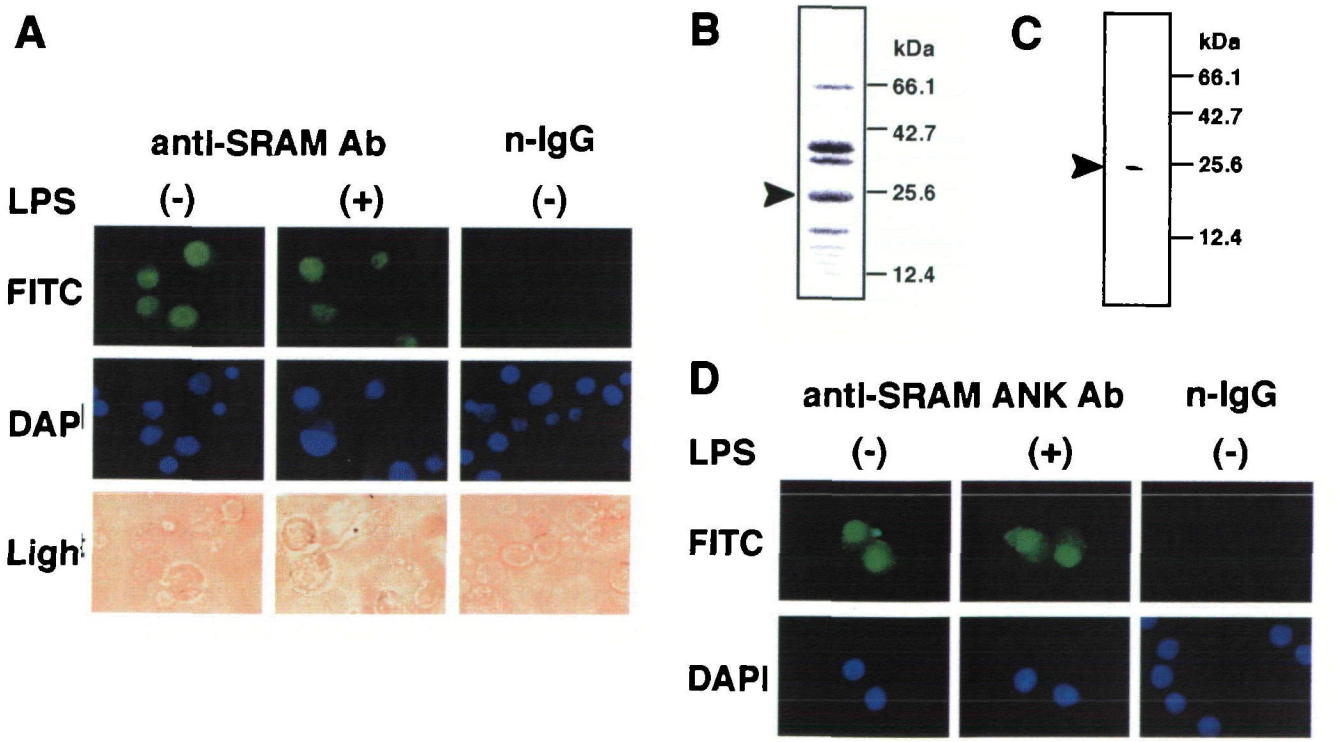


Fig. 3. **Immunohistochemistry of SRAM in NIH-Sape-4 cells with or without LPS treatment.** (A) NIH-Sape-4 cells were cultured in Grace's insect medium containing 5% FBS for 6 h in the presence (+) or absence (−) of LPS. They were then subjected to immunofluorescence staining with affinity-purified antibody against SRAM. FITC and DAPI indicate immunofluorescence and nuclear staining, respectively. Light shows the blight field. (B) SDS polyacrylamide gel electrophoretic profile of SRAM (50 μg) digested with trypsin. The arrowhead denotes the 24-kDa proteolytic fragment containing the ankyrin domain of SRAM. (C) Immunoblotting of the same digests (0.5 μg) with affinity purified anti-SRAM-ANK antibody. The band indicated by the arrowhead corresponds to the 24-kDa proteolytic fragment. (D) The same experiment as in (A), but with affinity-purified anti-SRAM-ANK antibody.

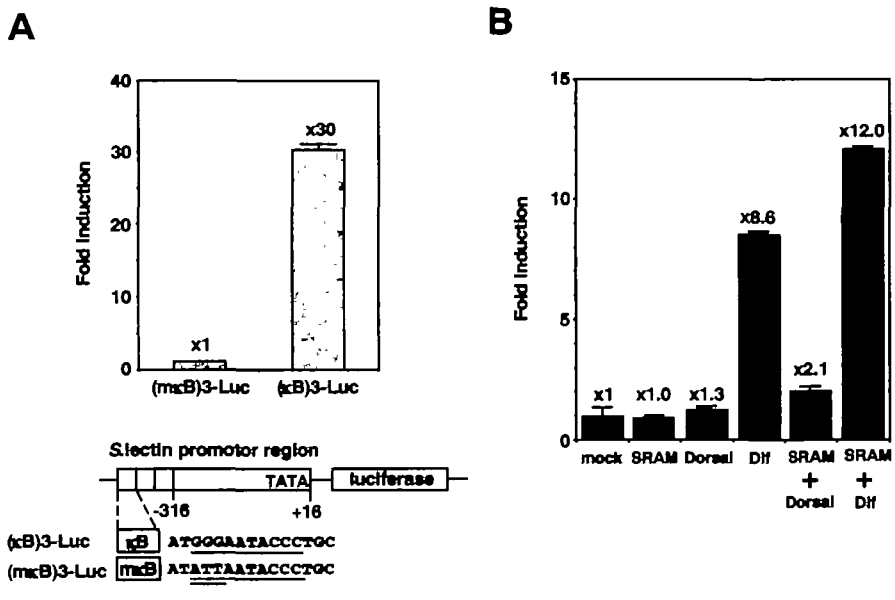


Fig. 4. **Transfection of the reporter gene containing the *Sarcophaga* lectin gene promoter.** (A) NIH-Sape-4 cells were transfected with the luciferase reporter gene under the control of the 5′ upstream regulatory element of the *Sarcophaga* lectin gene. The reporter constructs contained 3 tandem repeats of an intact κB motif of the *Sarcophaga* lectin gene (GGGAATACCC) or its mutated copy (ATTAATACCC), as illustrated at the bottom. Luciferase activity is indicated as *x*-fold levels of induction. (B) mbn-2 cells were transfected with SRAM, Dorsal, and Dif alone, or co-transfected with SRAM and Dorsal or SRAM and Dif together, with the reporter construct containing intact κB motifs. Luciferase activity is indicated by *x*-fold levels of induction compared with mock transfection.

mune protein genes in the fat body (data not shown).

Effect of SRAM on the Promoter Activity of the Sarcophaga Lectin Gene—Previously, we demonstrated that SRAM binds to the κB motif of the *Sarcophaga* lectin gene, and that the transcription of the *Sarcophaga* lectin gene in the nuclear extract of NIH-Sape-4 cells is specifically inhibited by the DNA fragment containing this motif (*24*). These results suggest that SRAM participates in the transcription of the *Sarcophaga* lectin gene by binding to the κB motif. We examined the effect of an anti-SRAM antibody on transcription of the *Sarcophaga* lectin gene in the nuclear extract of NIH-Sape-4 cells. However, no appreciable inhibition of the transcription of the *Sarcophaga* lectin gene was detected when anti-SRAM antibody was added to the reaction mixture (data not shown). So we performed luciferase assay using transfected NIH-Sape-4 cells. When luciferase reporter vectors driven by the 5′-upstream regulatory region of the *Sarcophaga* lectin gene containing 3 tandem repeats of the κB motif or its mutated one were transfected into NIH-Sape-4 cells, the reporter activity with intact κB motifs was about 30 times higher than that found with mutated ones (Fig. 4A). These results suggest that SRAM indeed participates in the expression of the *Sarcophaga* lectin gene in *Sarcophaga*.

We intended to examine the effect of transiently overexpressed SRAM in NIH-Sape-4 cells on the expression of the reporter gene containing intact κB motifs. However, overexpression of SRAM in these cells was technically very difficult. Therefore, we performed the same experiments using *Drosophila* malignant hemocyte line, mbn-2 cells (*40*), because an overexpression system of exogenous proteins driven by the *Drosophila* actin-5C promoter had been established in these cells (*28*). Overexpression of SRAM in these cells was confirmed by immunoblotting. No significant enhancement of luciferase activity was detected when SRAM alone was overexpressed (Fig. 4B). Considering the possibility that SRAM might function as a heterodimer with other Rel-family proteins, we performed a co-transfection assay with Dorsal or Dif. Dif alone gave an 8.6-fold increase in reporter activity, and co-transfection of Dif and SRAM enhanced reporter activity 12-fold compared with the level of mock transfection (Fig. 4B). Reporter activity with expression of Dorsal alone was almost the same as that with SRAM alone, but we detected a small increase in reporter activity with their co-transfection. As these results are reproducible, we assume that there is a positive interaction at least between SRAM and Dif, but that the interaction between SRAM and Dorsal is very weak, if it occurs at all. Almost the same results were obtained with *Drosophila* embryonic cell line SL-2 cells (data not shown).

DISCUSSION

Previously, we purified a 59-kDa protein that binds specifically to the κB motif of the *Sarcophaga* lectin gene (*24*). In the present study we isolated cDNA for the 59-kDa protein and found that it was a Rel/Ankyrin-family protein, renamed SRAM. When we compared the structures of *Drosophila* Relish and SRAM, the latter contained fewer ankyrin repeats and lacked a Ser-rich domain and a PEST sequence. The cleavage site of Relish that separates RHD and ankyrin repeats is present in this Ser-rich domain (*15*). The PEST sequence is believed to be required for metabolism and turnover of Relish. Moreover, unlike with Relish, much of SRAM seems to localize in nuclei as an intact protein, irrespective of immune stimuli. This is a unique feature of SRAM. No other Rel/Ankyrin-family protein is known to function in this way within nuclei. Usually, ankyrin repeats are cleaved off and RHD moves into the nuclei as a homodimers or heterodimers in response to various immune stimuli. Thus, SRAM is likely to be a novel member of Rel/Ankyrin-family.

In our previous paper, we demonstrated that the DNA-binding activity of SRAM in a nuclear extract of NIH-Sape-4 cells was clearly enhanced when the cells are treated with LPS in advance (*24*). Thus, SRAM in the nuclei seems to be activated *in situ* when the cells were treated with LPS. Generally, the redox potential of Rel protein is believed to alter its DNA-binding activity (*37*, *41*). However, the Cys residue that is assumed to be involved in redox reg-

ulation in Rel-family proteins is substituted for Ser in SRAM. Therefore, SRAM is likely to be activated by a mechanism other than redox regulation.

It is clear that the κB motif is important for the activation of the *Sarcophaga* lectin gene in NIH-Sape-4 cells, because the luciferase reporter vector driven by the 5′-upstream regulatory region of the *Sarcophaga* lectin gene containing 3 tandem repeats of the κB motif was expressed in these cells in a κB motif-dependent manner. As SRAM is the only protein so far known that binds to this motif in *Sarcophaga*, we assume that SRAM participates in the activation of the *Sarcophaga* lectin gene in NIH-Sape-4 cells. However, overexpression of SRAM alone did not enhance the expression of the same reporter gene in *Drosophila* mbn-2 cells. SRAM might require another factor that is present in NIH-Sape-4 cells, but not in mbn-2 cells, for the expression of this reporter gene. We co-expressed Dif or Dorsal with SRAM in mbn-2 cells and found that Dif alone was sufficient for the expression of the reporter gene. However, when Dif was co-expressed with SRAM, expression of the reporter gene was always enhanced. Unlike Dif, Dorsal alone did not seem to affect expression of the reporter gene. When Dorsal was co-expressed with SRAM, expression of the reporter gene tended to be enhanced. However, since the basal level of expression of the reporter gene with Dorsal or SRAM alone was about the same as that for mock transfection, and since the effect of co-transfection of Dorsal and SRAM was to double this level at most, it is difficult to assess the effect of the co-transfection of Dorsal and SRAM in this system.

As SRAM enhances the effect of Dif in mbn-2 cells, we speculate that SRAM functions cooperatively with the *Sarcophaga* homologue of Dif in the transcription of the *Sarcophaga* lectin gene. SRAM could also interact with *Sarcophaga* homologues of Dorsal, Relish and Gambif-1. Recently, it was reported that different combinations of Rel proteins control the activity of various immune protein genes (*17*). Another possibility is that SRAM interacts with transcription factors other than Rel-family proteins. In *Sarcophaga*, a factor named ATBP, which binds to the AT-stretches located in the 5′ upstream region and the first intron of the *Sarcophaga* lectin gene, has been identified (*22, 23*). STAT protein (*42, 43*) and GATA factor (*9*) are also known to be involved in the immune response of *Drosophila* and *Anopheles*.

Phylogenetic analysis suggests that SRAM is the closest Rel/Ankyrin-family protein to Relish. Hultmark *et al.* have discussed the possibility that the ancestral Rel-family protein contained both the RHD and ankyrin repeats like Relish, but that it eventually lost its ankyrin repeats to yield Dorsal and Dif (*15*). SRAM might have evolved from the same ancestral protein by loosing a part of its ankyrin repeats and gaining a novel activation mechanism.

We thank Drs. J.A. Hoffman and J.M. Reichhart for the Dorsal plasmid, and Dr. Y. Engstrom for the Dif plasmid. We also thank R. Steward, and D. Hultmark for reagents and/or discussions.

REFERENCES

1. Brey, P.T. and Hultmark, D. (1998) *Molecular Mechanisms of Immune Responses in Insects*, Chapman and Hall, London
2. Hoffmann, J.A. and Reichhart, J.M. (1997) *Drosophila* immunity. *Trends Cell Biol.* **7**, 309–316
3. Natori, S. (1994) Function of antimicrobial proteins in insects. *Ciba Founda. Symp.* **186**, 123–132
4. Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318
5. Kopp, E.B. and Medzhitov, R. (1999) The Toll-receptor family and control of innate immunity. *Curr Opin Immunol.* **11**, 13–18
6. Baeuerle, P.A. and Baltimore, D. (1996) NF-kappa B: ten years after. *Cell* **87**, 13–20
7. Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D., and Miyamoto, S. (1995) Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev.* **9**, 2723–2735
8. Ghosh, S., May, M.J., and Kopp, E.B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225–260
9. Engstrom, Y. (1999) Induction and regulation of antimicrobial peptides in *Drosophila*. *Dev. Comp. Immunol.* **23**, 345–358
10. Pahl, H.L. (1999) Activators and target genes of Rel/NF-kappa B transcription factors. *Oncogene* **18**, 6853–6866
11. Gilmore, T.D. (1999) The Rel/NF-kappa B signal transduction pathway: introduction. *Oncogene* **18**, 6842–6844
12. Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A., and Israel, A. (1990) The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. *Cell* **62**, 1007–1018
13. Steward, R. (1987) Dorsal, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-rel. *Science* **238**, 692–694
14. Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez-Crespo, S., Tatei, K., and Levine, M. (1993) Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763
15. Dushay, M.S., Asling, B., and Hultmark, D. (1996) Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **93**, 10343–10347
16. Barillas-Mury, C., Charlesworth, A., Gross, I., Richman, A., Hoffmann, J.A., and Kafatos, F.C. (1996) Immune factor Gambif1, a new rel family member from the human malaria vector, *Anopheles gambiae*. *EMBO J.* **15**, 4691–4701
17. Han, Z.S. and Ip, Y.T. (1999) Interaction and specificity of Rel-related proteins in regulating *Drosophila* immunity gene expression. *J. Biol. Chem.* **274**, 21355–21361
18. Geisler, R., Bergmann, A., Hiromi, Y., and Nusslein-Volhard, C. (1992) Cactus, a gene involved in dorsoventral pattern formation of *Drosophila*, is related to the I kappa B gene family of vertebrates. *Cell* **71**, 613–621
19. Kobayashi, A., Hirai, H., Kubo, T., Ueno, K., Nakanishi, Y., and Natori, S. (1989) Cloning and *in vitro* transcription of the *Sarcophaga* lectin gene. *Biochim. Biophys. Acta.* **1009**, 244–250
20. Kanai, A. and Natori, S. (1989) Cloning of gene cluster for sarcotoxin I, antibacterial proteins of *Sarcophaga peregrina*. *FEBS Lett.* **258**, 199–202
21. Kanai, A. and Natori, S. (1990) Analysis of a gene cluster for sarcotoxin II, a group of antibacterial proteins of *Sarcophaga peregrina*. *Mol. Cell. Biol.* **10**, 6114–6122
22. Matsui, M., Kobayashi, A., Kubo, T., and Natori, S. (1994) Purification and characterization of ATBP, a novel protein that binds to A/T stretches in three segments of the *Sarcophaga* lectin gene. *Eur. J. Biochem.* **219**, 449–454
23. Nakanishi-Matsui, M., Kubo, T., and Natori, S. (1995) Molecular cloning and nuclear localization of ATBP, a novel (A+T)-stretch-binding protein of *Sarcophaga peregrina* (flesh fly). *Eur. J. Biochem.* **230**, 396–400
24. Kobayashi, A., Matsui, M., Kubo, T., and Natori, S. (1993) Purification and characterization of a 59-kilodalton protein that specifically binds to NF-kappa B-binding motifs of the defense protein genes of *Sarcophaga peregrina* (the flesh fly). *Mol. Cell. Biol.* **13**, 4049–4056

25. Komano, H., Kasama, E., Nagasawa, Y., Nakanishi, Y., Matsuyama, K., Ando, K., and Natori, S. (1987) Purification of *Sarcophaga* (fleshfly) lectin and detection of sarcotoxins in the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*. *Biochem. J.* **248**, 217–222
26. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467
27. Homma, K., Matsushita, T., and Natori, S. (1996) Purification, characterization, and cDNA cloning of a novel growth factor from the conditioned medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina* (flesh fly). *J. Biol. Chem.* **271**, 13770–13775
28. Krasnow, M.A., Saffman, E.E., Kornfeld, K., and Hogness, D.S. (1989) Transcriptional activation and repression by Ultrabithorax proteins in cultured *Drosophila* cells. *Cell* **57**, 1031–1043
29. Thisse, C., Perrin-Schmitt, F., Stoetzel, C., and Thisse, B. (1991) Sequence-specific transactivation of the *Drosophila* twist gene by the dorsal gene product. *Cell* **65**, 1191–1201
30. Lin, L. and Ghosh, S. (1996) A glycine-rich region in NF-kappa B p105 functions as a processing signal for the generation of the p50 subunit. *Mol. Cell. Biol.* **16**, 2248–2254
31. Rogers, S., Wells, R., and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364–368
32. Muller, C.W., Rey, F.A., Sodeoka, M., Verdine, G.L., and Harrison, S.C. (1995) Structure of the NF-kappa B p50 homodimer bound to DNA. *Nature* **373**, 311–317
33. Ghosh, G., van Duyne, G., Ghosh, S., and Sigler, P.B. (1995) Structure of NF-kappa B p50 homodimer bound to a kappa B site. *Nature* **373**, 303–310
34. Chen, F.E., Huang, D.B., Chen, Y.Q., and Ghosh, G. (1998) Crystal structure of p50/p65 heterodimer of transcription factor NF-kappa B bound to DNA. *Nature* **391**, 410–413
35. Cramer, P., Varrot, A., Barillas-Mury, C., Kafatos, F.C., and Muller, C.W. (1999) Structure of the specificity domain of the Dorsal homologue Gambif1 bound to DNA. *Struct. Fold Des.* **7**, 841–852
36. Chen, F.E. and Ghosh, G. (1999) Regulation of DNA binding by Rel/NF-kappa B transcription factors: structural views. *Oncogene* **18**, 6845–6852
37. Hayashi, T., Ueno, Y., and Okamoto, T. (1993) Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. *J. Biol. Chem.* **268**, 11380–11388
38. Qin, J., Clore, G.M., Kennedy, W.M., Huth, J.R., and Gronenborn, A.M. (1995) Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NF-kappa B. *Structure* **3**, 289–297
39. Mercurio, F. and Manning, A.M. (1999) NF-kappa B as a primary regulator of the stress response. *Oncogene* **18**, 6163–6171
40. Gateff, E., Gissmann, L., Shrestha, R., Plus, N., Pfister, H., Schroder, J., and Zur Hansen, H. (1980) *Invertebrate Systems In Vitro* (Kurstak, E., Maramarosch, K., and Dubendorfer, A., eds.) pp. 517–533, Elsivier, North Holland Biochemical Press
41. Matthews, J.R., Wakasugi, N., Virelizier, J.L., Yodoi, J., and Hay, R.T. (1992) Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res.* **20**, 3821–3830
42. Barillas-Mury, C., Han, Y.S., Seeley, D., and Kafatos, F.C. (1999) *Anopheles gambiae* Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection. *EMBO J.* **18**, 959–967
43. Yan, R., Small, S., Desplan, C., Dearolf, C.R., and Darnell, J.E., Jr. (1996) Identification of a Stat gene that functions in *Drosophila* development. *Cell* **84**, 421–430