A novel C-type lectin that agglutinates rabbit red cells was purified from NIH-Sape-4 cells derived from the flesh fly (Sarcophaga peregrina), and its cDNA was isolated. This lectin, named granulocytin, appeared to be a trimer of a 20-kDa subunit consisting of 151 amino acid residues. The gene for granulocytin was activated in the larval body wall, and its expression was enhanced when the larval body wall was injured. In third instar larvae, granulocytin was found to be synthesized by hemocytes and secreted into the hemolymph. The molecular mass and gene expression patterns of granulocytin were very similar to those of Drosophila lectin that we reported previously (Haq, S., Kubo, T., Kurata, S., Kobayashi, A., and Natori, S. (1996) J. Biol. Chem. 271, 20213–20218). However, these two lectins showed amino acid identities of 20% at most, and no significant hapten sugar for granulocytin was identified.

Insects respond to microbial infection through cellular and humoral defense mechanisms and are known to secrete various antimicrobial proteins and lectins into their hemolymph in response to bacterial challenges and body injury (1–8). Most of these humoral defense proteins are synthesized by the fat body (9–12) and some by hemocytes (13). Although several antimicrobial proteins have been isolated from various insects, sequenced, and characterized (14), the primary sequences of very few insect lectins have been determined.

In a previous study, we purified a galactose-binding C-type lectin from the hemolymph of immunized larvae of Sarcophaga peregrina (flesh fly) and characterized it (15–17). This lectin (Sarcophaga lectin) was a large molecule with a molecular mass of 190 kDa, consisting of 32- and 30-kDa subunits in a molar ratio of 2:1. These two subunits were essentially the same protein derived from a single gene, and their difference in size was due to glycosylation. Sarcophaga lectin was found to be needed for the elimination of sheep red cells introduced into the larval body cavity (18). It was also found to play roles in imaginal disc differentiation, suggesting that Sarcophaga lectin functions in both defense and development of this insect (19–21).

To study the biological role of Sarcophaga lectin in the development of this insect, we identified its specific binding protein with a molecular mass of 10 kDa (22). This binding protein was isolated from the membrane fraction of NIH-Sape-4 cells, an embryonic cell line of Sarcophaga, and was shown to be distributed heterogeneously on the surface of imaginal discs (22). Incidentally, we found that a protein that reacted with a monoclonal antibody raised against the Sarcophaga lectin-binding protein was a novel C-type lectin of this insect. In this paper, we report the purification, characterization, and cDNA cloning of this new lectin named granulocytin.

**MATERIALS AND METHODS**

**Animals and Collection of Hemocytes, Hemolymph, and Fat Bodies—**

Sarcophaga embryonic cell line, NIH-Sape-4, was used throughout (24). NIH-Sape-4 cells were cultured in M-M medium (120 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl₂, 0.5 mM MgCl₂, 1.6 mM NaH₂PO₄, 1.4 mM NaHCO₃, 4 mg/ml glucose, 5 mg/ml yeast extract (DIFCO), 6.5 mg/ml bovine serum albumin (DIFCO) (25), and the and conditioned medium were harvested exactly as described previously (24).

**Monoclonal Antibody SLB1 and Preparation of SLB1-Sepharose—**

A monoclonal anti-Sarcophaga lectin-binding protein antibody, SLB1, was raised as reported previously (22). SLB1-Sepharose was prepared by coupling 20 mg of SLB1 with 5 g of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech), according to the manufacturer’s instructions, suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), and stored at 4 °C until required for use.

**Purification of a 20-kDa Protein That Reacts with SLB1 from NIH-Sape-4 Cells—**

NIH-Sape-4 cells (20 g) were suspended in 400 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 100 mM galactose and homogenized in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 100 × g for 1 min to remove the cell debris, and the supernatant was centrifuged at 100,000 × g for 30 min. The resulting precipitate was washed twice by repeating centrifugation, suspended in 200 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.5% (w/v) Nonidet P-40, kept for 1 h at 4 °C, and centrifuged at 100,000 × g for 30 min. The resulting supernatant was used as the solubilized protein fraction.

The solubilized protein was diluted 4-fold with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and applied to an SLB1-Sepharose column (1 × 5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.125% (w/v) Nonidet P-40. The column was washed well, and the adsorbed material was eluted with the above buffer containing 8 mM urea. This fraction was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 40 mM octylglucoside and 150 mM NaCl, concentrated to about 500 ml, and then subjected to fast protein liquid chromatography on a Mono Q column (Amersham Pharmacia Biotech). A protein that reacted with SLB1 was recovered in the flow-through fraction. At this stage, the protein was pure enough to yield a single 20-kDa band after SDS-polyacrylamide gel electrophoresis. The protein concentration was measured by the method of Lowry et al. (26) using bovine serum albumin as the standard.

**Cloning and Sequencing of the 20-kDa Protein cDNA—**

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) AB006076.

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was raised against the 20-kDa protein and affinity-purified using the method described previously (27, 28). About 10,000 clones of an NIH-Sape-4 cDNA library (29, 30) were screened with this affinity-purified antibody and the alkaline phosphatase-conjugated secondary antibody (Bio-Rad). For nucleotide sequencing of the cDNA, the insert DNA was subcloned into plasmid script (Stratagene) and sequenced by the dideoxy chain termination method using a T7 dye terminator sequencing kit (Applied Biosystems). The nucleotide sequences of both strands were determined.

Northern Blot Analysis—Northern blot hybridization was performed using a reaction mixture comprised of 50% formamide, 5% SSPE (1× SSPE contains 0.15 M NaCl, 10 mM NaPO4, 1 mM EDTA), 1× Denhardt’s solution (0.02% (w/v) each of Ficoll-400, bovine serum albumin, and polyvinylpyrrolidone), 0.1% SDS and sonicated salmon sperm DNA solution (200 μg/ml) for 18 h at 42 °C. Then, the filters were washed for 30 min each with 2× SSC (1× SSC contains 0.15 M NaCl, 0.05 M sodium citrate) containing 0.1% SDS at room temperature and 42 °C and finally autoradiographed at −80 °C. The DNA used as a probe was the 20-kDa protein cDNA labeled with [α-32P]dCTP using a random primer labeling kit (Takara, Tokyo).

Electrophoresis and Immunoblotting—Electrophoresis on SDS-polyacrylamide slab gel was carried out by the method of Laemmli (31). Samples were denatured by boiling them for 5 min in 1% SDS containing 2% (v/v) 2-mercaptoethanol, and after electrophoresis, the gel was stained using the method of Fairbanks et al. (32). For immunoblotting, the proteins separated by electrophoresis were transferred electrophoretically from the gel onto filters (Immobilon-P, Millipore), which were immersed in 20 mM Tris-HCl buffer (pH 7.9) containing 5% (w/v) skim milk for 1 h. After washing with a rinsing solution (10 mM Tris-HCl buffer, pH 7.9, containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.01% (w/v) sodium azide, and 0.25% skim milk), they were dipped in rinsing solution containing the affinity-purified antibody against the 20-kDa protein and kept for 1 h. Then, they were washed well with rinsing solution, transferred to 5 ml rinsing solution containing radioiodinated anti-rabbit IgG (18.5 kBq), and kept at room temperature for 1 h. Finally, they were washed well with rinsing solution, dried, and subjected to autoradiography using Kodak XAR film.

Assay of Hemagglutinating Activity—Rabbit red blood cells (RRBC)1 were washed well and suspended in buffered insect saline. To measure hemagglutinating activity, 25 μl of serially diluted sample solution was mixed with 25 μl of 2.5% (w/v) RRBC suspension in a well of a V-bottomed microtiter plate, and then incubated at 37°C for 1 h. Agglutinated RRBC formed a diffuse mat, whereas unagglutinated RRBC sedimented and culture medium of NIH-Sape-4 cells were subjected to immunoblotting and cDNA cloning. Immunoblotting of the 20-kDa protein. The gel and filter was calibrated as described in the legend to Fig. 1.

RESULTS

Affinity Purification of 20-kDa Protein from NIH-Sape-4 Cells—In a previous study, we isolated a Sarcophaga lectin-binding protein from NIH-Sape-4 cells and raised a monoclonal antibody, named SLB1, against it as described before (22). To purify the Sarcophaga lectin-binding protein in quantity from the membrane fraction of NIH-Sape-4 cells, we performed affinity chromatography using SLB1-conjugated Sepharose CL-4B followed by fast protein liquid chromatography on a Mono Q column. The protein thus obtained yield a broad band with a molecular mass of 20 kDa after SDS-polyacrylamide gel electrophoresis (Fig. 1). Unlike the 10-kDa Sarcophaga lectin-binding protein, this 20-kDa protein showed essentially no Sarcophaga lectin binding activity, indicating that it is not the Sarcophaga lectin-binding protein we characterized before (22). The molecular mass of the intact protein, estimated from the elution volume from a molecular sieving column of Suparose 12, was 60 kDa, suggesting that the intact protein is a trimer of the 20-kDa subunit. About 100 μg of 20-kDa protein was obtained from 4 × 1011 NIH-Sape-4 cells.

To characterize this 20-kDa protein further, we raised a polyclonal antibody against the purified protein, affinity-purified it and used it for immunoblotting and cDNA cloning. Immunoblotting revealed that not only NIH-Sape-4 cells, but also their culture medium contained this 20-kDa protein (Fig. 2A). The immunoreactive proteins in the culture medium that migrated faster than the 20-kDa protein were probably its degradation products. These results suggest that the 20-kDa protein is not a membrane protein, but a secretory protein, which was confirmed by detecting the 20-kDa protein in hemolymph. As shown in Fig. 2B, this protein was detected in hemocytes, hemolymph, and fat bodies from third instar larvae, suggesting that this protein is a hemolymph protein, produced possibly by

1 The abbreviations used are: RRBC, rabbit red blood cells; CRD, carbohydrate recognition domain.
residues. Two possible sequences consisting of 151 residues and its leader peptide consisting of 22 residues, APLTKWFKTDNNT, which we determined separately, were also present in this protein. Therefore, we concluded that this is cDNA for the 20-kDa protein.

The nucleotide and deduced amino acid sequences encoded in this cDNA are shown in Fig. 3. The partial amino-terminal residue of the hemocytes and fat body of third instar larvae.

**Isolation of cDNA for the 20-kDa Protein**—To determine the amino acid sequence of the 20-kDa protein, we tried to isolate its cDNA by screening an NIH-Sape-4 cDNA library with the affinity-purified antibody. Three positive clones had identical nucleotide sequences, and the protein encoded in this cDNA was shown to encode the 20-kDa protein with trypsin: WFK, LITLK, TYREDELLSQYLK, QTY-

**Immunofluorescence Study of the 20-kDa Protein**—As the 20-kDa protein was found to contain four Cys residues that are conserved in the CRDs of many C-type lectins (36), suggesting that the 20-kDa protein is a lectin, but it lacked two of three residues, Glu and Asp, which are reported to be responsible for sugar and calcium binding (37, 38). The molecular mass of the 20-kDa protein was about the same as that of Drosophila lectin.

**Hemagglutinating Activity of the 20-kDa Protein**—As the 20-kDa protein was found to be structurally related to Sarcophaga and Drosophila C-type lectins, we examined its hemagglutinating activity using various red cells in the presence of 1 mM CaCl₂. No agglutination of sheep, pig, chicken, horse, guinea pig, bovine, or human red cells was observed, but significant agglutination of RRBC did occur. Calcium was essential for this hemagglutinating activity, indicating that the 20-kDa protein is a C-type lectin. Next we examined the effects of various carbohydrates on the hemagglutinating activity of the 20-kDa protein. No specific hapten sugar has been identified in the monosaccharides tested so far. Glucose, galactose, fucose, xylose, rhamnose, N-acetylmuramic acid, and N-acetylneuraminic acid (all at 100 μg/ml) inhibited the hemagglutinating activity by 50%. Bovine submaxillary gland mucin (10 μg/ml) inhibited the hemagglutinating activity, but porcine stomach mucin had no inhibitory effect. Lipopolysaccharide from Escherichia coli 0111:B4 was a weak inhibitor (100 μg/ml) but the other lipopolysaccharides tested showed no inhibitory effects. This carbohydrate specificity was clearly different from those of Sarcophaga and Drosophila lectins, the hapten sugars of which were identified as galactose (15, 34).

**Expression of the Granulocytin Gene**—Immunoblotting detected granulocytin both in hemocytes and fat bodies of third instar larvae. However, as shown in Fig. 7A, Northern blotting detected 0.9 kilobase mRNA for granulocytin exclusively in the hemocytes and fat body of third instar larvae.

**Fig. 3.** Nucleotide sequence of cDNA for the 20-kDa protein. Nucleotide numbers are shown above the nucleotide sequence, and the numbers of the amino acid residues, starting from the first Met, are given to the right of each line. The partial amino acid sequences determined are underlined, possible N-glycosylation sites are boxed, the amino-terminal residue is indicated by an arrow, and the asterisk and double underlining denote the termination codon and poly(A) additional signal, respectively.

As the 20-kDa protein was found to be a novel C-type lectin of Sarcophaga and Drosophila C-type lectins, we examined its hemagglutinating activity using various red cells in the presence of 1 mM CaCl₂. No agglutination of sheep, pig, chicken, horse, guinea pig, bovine, or human red cells was observed, but significant agglutination of RRBC did occur. Calcium was essential for this hemagglutinating activity, indicating that the 20-kDa protein is a C-type lectin. Next we examined the effects of various carbohydrates on the hemagglutinating activity of the 20-kDa protein. No specific hapten sugar has been identified in the monosaccharides tested so far. Glucose, galactose, fucose, xylose, rhamnose, N-acetylmuramic acid, and N-acetylneuraminic acid (all at 100 μg/ml) inhibited the hemagglutinating activity by 50%. Bovine submaxillary gland mucin (10 μg/ml) inhibited the hemagglutinating activity, but porcine stomach mucin had no inhibitory effect. Lipopolysaccharide from Escherichia coli 0111:B4 was a weak inhibitor (100 μg/ml) but the other lipopolysaccharides tested showed no inhibitory effects. This carbohydrate specificity was clearly different from those of Sarcophaga and Drosophila lectins, the hapten sugars of which were identified as galactose (15, 34).

**Expression of the Granulocytin Gene**—Immunoblotting detected granulocytin both in hemocytes and fat bodies of third instar larvae. However, as shown in Fig. 7A, Northern blotting detected 0.9 kilobase mRNA for granulocytin exclusively in
hemocytes, not in the fat body, indicating that this lectin was synthesized by the hemocytes.

Expression of the granulocytin gene was examined at various developmental stages of *Sarcophaga*. No significant expression was detected throughout the embryonic stage to the second instar larval stage, but this gene was expressed in third instar larvae and the expression continued until the early pupal stage. Then, this gene was turned off during the rest of the pupal stage and was activated again in the newly emerged adult flies (Fig. 7B). We examined the post-injury acute-phase expression of the granulocytin gene in hemocytes from third instar larvae. As shown in Fig. 7C, expression of this gene was enhanced 6 h after pricking the larval body wall with a thin needle and declined slightly 18 h later. These expression patterns were very similar to those of the *Drosophila* lectin gene (34).

**DISCUSSION**

In this study, we identified a novel C-type lectin of *Sarcophaga* and purified it from the membrane fraction of NIH-Sape-4 cells by SLB1-Sepharose chromatography. SLB1 is a monoclonal antibody against *Sarcophaga* lectin-binding protein with a molecular mass of 10 kDa (22). As this novel lectin (granulocytin) clearly differed from the *Sarcophaga* lectin-binding protein, SLB1 should have affinity for both the *Sarcophaga* lectin-binding protein and granulocytin. The epitope for SLB1 in the *Sarcophaga* lectin-binding protein may be a specific carbohydrate chain, which granulocytin may also contain. In which case, why was only granulocytin recovered from the SLB1-Sepharose column? Granulocytin content of NIH-Sape-4 cells is probably an order of magnitude higher than that of the *Sarcophaga* lectin-binding protein and, therefore, the former protein was trapped selectively by the column.

We solubilized granulocytin from the membrane fraction of NIH-Sape-4 cells with octylglucoside as described before (22). As granulocytin turned out to be a soluble hemolymph protein, hemolymph would probably have been a better source for purification. We might have purified granulocytin contaminating the membrane fraction or the granules that store granulocytin.
might have been recovered in the membrane fraction we prepared. Indeed, we found that the latter is the case. The purified 20-kDa protein yielded a broad band after SDS-polyacrylamide gel electrophoresis and sometimes yielded a doublet band. As the amino-terminal sequences of the proteins from these two bands were identical, their different electrophoretic mobilities were probably due to differences in their carbohydrate chains.

Of the seven types of red cells tested, only those from rabbits were agglutinated by this lectin possibly because this lectin recognizes a specific sequence of carbohydrates present only in RRBC. Granulocytin appears to be the Sarcophaga counterpart of Drosophila lectin because their molecular masses and gene expression patterns during development and expression during the acute-phase response to injury are very similar (34). However, the carbohydrate specificities of these two lectins are different. At present, the biological role of granulocytin is unknown. However, as it is synthesized prior to pupation, production of this lectin is probably prerequisite for metamorphosis.

The Northern blotting analysis findings suggest that expression of the granulocytin gene is involved in the acute-phase response to injury, as is Drosophila lectin gene expression (34). These lectins may be involved in the defense systems that eliminate invading parasites as well as in the process that disintegrates unnecessary larval tissues during metamorphosis. Our results demonstrated clearly that granulocytin is synthesized exclusively by hemocytes. We detected a positive signal of granulocytin in the fat body by immunoblotting. This signal may have been due to hemocytes contaminating the fat body preparation.

Five of the seven residues commonly found in the CRDs from various C-type lectins are conserved in the structure of the putative CRD of granulocytin. Of these seven residues, Glu, Asp, and Asn (see Fig. 5) were recently reported to be responsible for sugar and calcium binding (37, 38). Although the CRD of Drosophila lectin contains these three residues (34), that of granulocytin contains only Asn. The hapten sugar of Drosophila lectin was identified as galactose, but we could not identify that of granulocytin because many sugars at relatively high concentrations inhibited the hemagglutinating activity of granulocytin. The deficiency of these two residues might explain the broad and weak carbohydrate specificity of granulocytin.

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A Novel Lectin from Sarcophaga: ITS PURIFICATION, CHARACTERIZATION, AND cDNA CLONING
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