Distinct Cellular Interactions of Secreted and Transmembrane Ebola Virus Glycoproteins

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The mechanisms by which Ebola virus evades detection and infects cells to cause hemorrhagic fever have not been defined, though its glycoprotein, synthesized in either a secreted or transmembrane form, is likely involved. Here the secreted glycoprotein was found to interact with neutrophils through CD16b, the neutrophil-specific form of the Fc γ receptor III, whereas the transmembrane glycoprotein was found to interact with endothelial cells but not neutrophils. A murine retroviral vector pseudotyped with the transmembrane glycoprotein preferentially infected endothelial cells. Thus, the secreted glycoprotein inhibits early neutrophil activation, which likely affects the host response to infection, whereas binding of the transmembrane glycoprotein to endothelial cells may contribute to the hemorrhagic symptoms of this disease.

Ebola virus has been identified as the cause of several highly lethal outbreaks of hemorrhagic fever. Infection typically begins with flu-like symptoms, which often progress rapidly to fatal complications of hemorrhage, fever, and hypotensive shock (*1–4*). The negative-stranded genome of Ebola virus contains seven structural and regulatory proteins (5), but despite its relative simplicity, the molecular basis for Ebola virus pathogenicity is unknown. Among the viral gene products, the glycoprotein is found in two forms: a secreted form, 50 to 70 kD (6), synthesized in large amounts early in the course of infection, and an alternative transmembrane form, which arises from RNA editing to encode a 120- to 150-kD glycoprotein that is incorporated into the virion (6, 7). The first 295 amino acids of both proteins are identical in the Zaire strain, but secreted glycoprotein (sGP) contains an additional 69 and the transmembrane glycoprotein (GP) another 381 COOH-terminal amino acid residues (6). The specific cellular targets of these related gene products and their roles in the pathogenesis of Ebola infection have not been characterized.

To determine the specificity of Ebola virus glycoproteins, we transfected expression vectors encoding either sGP, GP, or a plasmid control (8) into human 293 cells, and cell culture supernatants were used as a source of relevant recombinant glycoproteins. Binding of sGP was determined by immunofluorescence analysis after incubation of the relevant supernatants with normal or transformed human cell lines. No binding was detected to several hematopoietic lineages, including lymphocytes or monocytes (Fig. 1A), transformed Jurkat or CEM T leukemias, or the HL-60 myelomonocytic or U-937 promonocytic leukemia cells (9). In contrast, sGP bound to granulocytic cells, as evidenced by fluorescence-activated cell sorting (FACS) analysis of this subset of peripheral blood mononuclear cells (PBMCs) discriminated by cell size and granularity (Fig. 1A). This cell specificity was confirmed by using double staining with a granulocyte-specific cell surface marker, CD15 (Fig. 1B). Absorption of sGP by purified neutrophils in the absence of antibodies also resulted in depletion of sGP, indicating that binding to the neutrophil occurred in the absence of antibody (Fig. 1C).

A potential structural similarity between Ebola GP and avian sarcoma virus envelope protein was previously proposed

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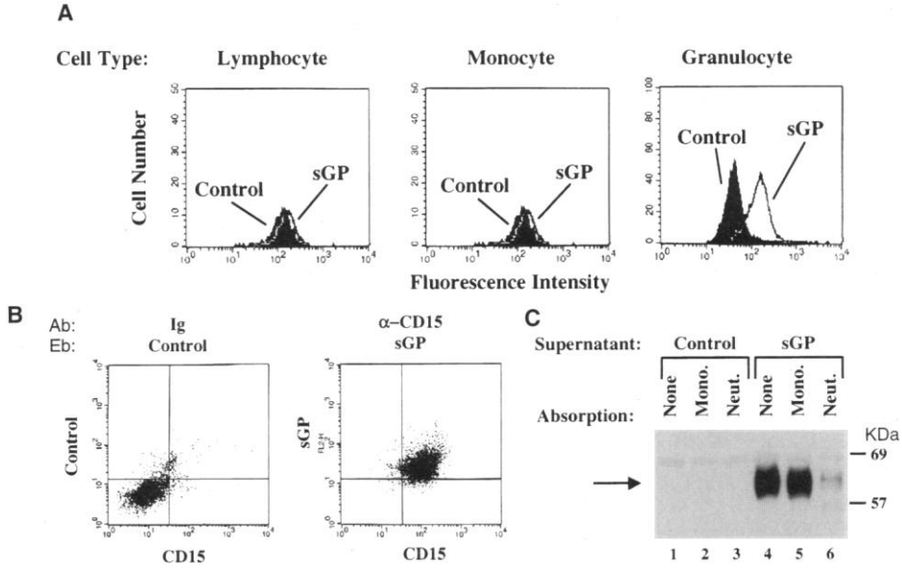

Fig. 1. Binding of sGP to neutrophils. (**A**) PBMCs from normal volunteers were incubated with control or sGP supernatants derived from transfected 293 cells, and immunostaining was performed with a rabbit antibody to sGP (anti-sGP, 1:1000) as described (*6, 8*). Secondary staining was performed with a fluorescein isothiocyanate (FITC)–conjugated goat antibody to rabbit immunoglobulin G (IgG, 1:64) (Sigma, F9887). All incubations were performed at 4°C for 30 min with comparable amounts of the relevant antibodies per 10^6 cells in a 50-μl volume. (**B**) Double immunostaining with anti-sGP and the neutrophil-specific marker CD15. Cells were incubated with a FITC-conjugated mouse antibody to human CD15 (α-CD15) (Caltag, MHCD1501), followed by secondary staining with a phycoerythrin-conjugated antibody to rabbit IgG (Sigma) to detect sGP binding. Cells were washed with phosphate-buffered saline (PBS), fixed in 1% formaldehyde, and analyzed by FACS. Ab, antibody; Eb, Ebola protein. (**C**) Specific absorption of sGP by neutrophils. Control or sGP supernatants derived from relevant transfected 293 cells (*8*) were incubated at 1:500 dilution with 10^6 mononuclear (Mono.) or granulocytic cells (Neut.). Cells were removed and the resulting supernatants analyzed by an 8% SDS–polyacrylamide gel electrophoresis gel. Protein immunoblot analysis was performed as described (*8*) with a rabbit antisera to sGP (1:5000) and a secondary antibody, horseradish peroxidase–conjugated donkey antibody to rabbit IgG at a dilution of 1:5000 (Amersham, NA934). Primary antibody was incubated for 30 min at room temperature, as was the secondary antibody. The immunocomplexes were detected by chemoluminescence with Supersignal chemiluminescent substrate reagents (Pierce) according to the manufacturer's instructions. Arrow indicates sGP-reactive band.

(*10*), raising the possibility that this protein could be incorporated into retroviral particles. To determine the binding specificity of the GP, we pseudotyped a Moloney leukemia virus. The infectivity of different cell types by this pseudotyped vector was determined with a luciferase reporter gene (*11*). This analysis revealed infection of cells different from those that interacted with sGP (Fig. 2, A and B). For example, though it could infect other cell types, transduction by the GP retroviral vector readily occurred in endothelial cells, either from the microvasculature (MVEC) or umbilical veins (HUVEC) (Fig. 2B), which did not bind sGP (Fig. 2C, left). When the specificity of GP retrovirus was compared with murine retroviruses pseudotyped with amphotropic or ecotropic envelope gp70 proteins, the range of susceptible target cells differed (Fig. 2B), suggesting that the virus receptor or receptors for Ebola GP differ from those previously described for gp70. Minimal binding of GP virus was observed on neutrophils, despite the ability of these cells to bind sGP (Fig. 2C, upper panel) and the fact that immunoreactive protein was detected on the virus (9). Conversely, GP virus binding to endothelial cells was readily detected, though these cells did not bind sGP (Fig. 2C, lower panel). When sGP was analyzed for its effect on GP retroviral gene transfer, infection was not inhibited by sGP (Fig. 2D), further confirming the divergent specificities of the two forms of the viral glycoprotein. Recent studies have revealed that the biochemical forms of these proteins differ, with sGP present in solution primarily as a homodimer and GP as a trimer (*12*), suggesting that differences in multimer composition may contribute to these alternative specificities.

Potential cell surface receptors for sGP were analyzed with antibodies to several neutrophil cell surface antigens to interfere with sGP binding, including CD15, L-selectin (CD62L), CD16b, and several common leukocyte antigens. Only the antibody to the neutrophil-specific form of the low-affinity Fc γ receptor III, CD16b, inhibited sGP binding specifically. Antibodies to CD62L, for example, did not inhibit sGP binding (Fig. 3). Binding to neutrophils correlated with their activation state and CD16b expression because no binding was observed in cells stimulated with phorbol 12-myristate 13-acetate (PMA) for 30 min, at which time CD16b expression was markedly decreased on these cells (Fig. 3, lower panel). Overexpression of this form of CD16 on a heterologous cell type, 3T3 fibroblasts, did not confer sGP binding to these cells according to FACS analysis (9), suggesting that CD16b is necessary but not sufficient for stable binding.

Binding of sGP did not inhibit neutrophil activation in response to potent pleiotropic activators (PMA, interleukin-8, or f-Met-Leu-Phe), as measured by down-modulation of L-selectin expression with FACS analysis (9). In a defined serum-free medium, partial activation of neutrophils was observed, with a decrease in L-selectin expression at 4 hours (Fig. 4). Under these conditions, incubation of neutrophils with sGP supernatant prevented this decrease in L-selectin expression (Fig. 4). Because L-selectin was not required for sGP binding (Fig. 3), this effect was apparently indirect, through a mechanism not yet defined, possibly involving CD16b or carbohydrate in-

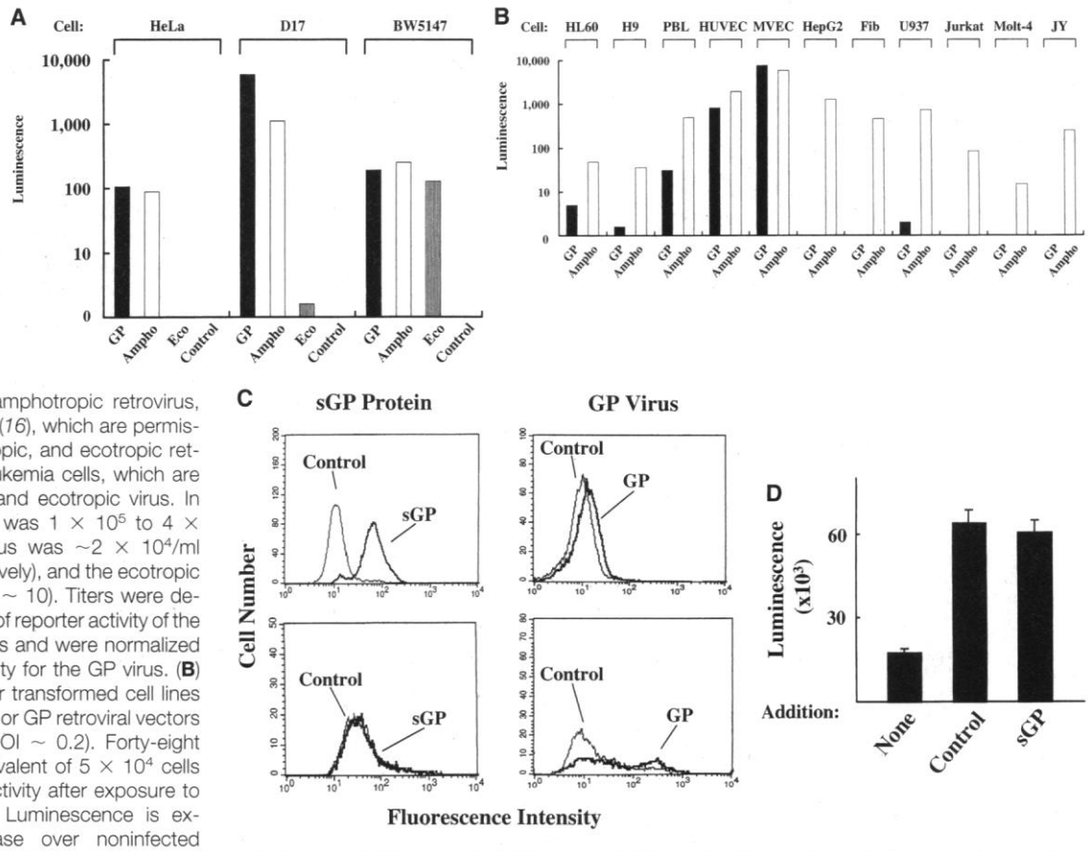

Fig. 2. Infection of different cell types by GP-pseudotyped retroviral vector and preferential binding to endothelial cells. (**A**) Infection of different indicator cell lines with the Ebola GP-pseudotyped retrovirus expressing luciferase (*15*). Amphotropic (Ampho) and ecotropic (Eco) retroviral vectors were used as controls. Viruses were diluted to different multiplicities of infection (MOIs) to provide for equal luciferase activity on HeLa cervical epithelial cells, permissive for amphotropic retrovirus, D17 dog osteosarcoma cells (*16*), which are permissive for amphotropic, xenotropic, and ecotropic retroviruses, and BW5147 T leukemia cells, which are permissive for amphotropic and ecotropic virus. In these groups, GP virus titer was 1×10^5 to 4×10^5/ml and amphotropic virus was $\sim 2 \times 10^4$/ml (MOIs ~ 1.0 and 0.1, respectively), and the ecotropic virus titer was $\sim 10^6$/ml (MOI ~ 10). Titers were determined by endpoint dilution of reporter activity of the amphotropic virus in D17 cells and were normalized to reverse transcriptase activity for the GP virus. (**B**) Analysis of different normal or transformed cell lines by infection with amphotropic or GP retroviral vectors at the same titer (10^4/ml, MOI ~ 0.2). Forty-eight hours after infection, an equivalent of 5×10^4 cells was assayed for luciferase activity after exposure to equal titers of viral stocks. Luminescence is expressed as the fold-increase over noninfected control cells. (**C**) The binding of sGP (left) or GP-pseudotyped retrovirus (right) to neutrophils (upper panel) or microvascular endothelium (lower panel) was determined by FACS. sGP binding was performed as in Fig. 1A, and retrovirus incubation was performed at 37°C for 2 hours in the presence of polybrene (8 μg/ml). (**D**) Infection of D17 cells by GP-pseudotyped virus in the absence (lane 1, none) or presence of control (lane 2) or sGP supernatant (lane 3) from transfected 293 cells. Gene transfer was measured by the luciferase assay (*15*). Luminescence refers to relative light units in the luciferase assay.

teractions of the highly glycosylated sGP protein.

The expression of alternative Ebola virus glycoproteins in clinical infection has long been recognized, but their functional roles and cell specificity have not been defined. Early after infection, large amounts of the secreted protein are found in the serum and precede fulminant replication and dissemination of the virus systemically, at which time synthesis of transmembrane GP is markedly increased (6). Here we found that the binding specificities of these two molecules differ. It had been proposed that sGP may serve as a decoy to prevent recognition of GP, possibly to temporarily inhibit virus binding to target cells. Our results suggest that this hypothesis is unlikely to be correct. The binding specificities of these proteins differ, and despite the fact that they are derived from the same viral gene, alternative forms of the glycoprotein have been selected for different functions.

Although these proteins share identical NH_2-terminal sequences, their COOH-terminal regions differ (5). These sequences are likely responsible for the differences in binding specificity, either through direct interactions in these domains or by their effect on multimerization. The secreted glycoprotein binds to neutrophils to prevent early events in activation, possibly serving to diminish any inflammatory responses that might provide innate immunity to the virus, facilitating productive viral replication. The subsequent increase in GP synthesis gives rise to virus that in turn could infect other cells. Filoviruses have been shown to infect and replicate in different cell types, and appear to grow readily in endothelial cells in vivo (*4*, *13*). Our findings suggest that virus's tropism for this cell type is probably determined by the specificity of GP. In fact, GP may be useful in targeting gene transfer vectors to endothelium for experimental or therapeutic purposes. In Ebola infection, preferential binding and infection of microvascular endothelial cells may lead ultimately to a loss of capillary integrity that results in the severe hemorrhage observed in the terminal stages of hemorrhagic fever. The differential binding of these two gene products from the same viral structural gene generated by RNA editing suggests that they have evolved functionally to differentially affect immunity and infectivity. The ability to facilitate viral replication and target the virus to endothelial cells by alternative products of the same viral gene represents an efficient genetic mechanism that can account for different pathologic features of this disease. Inhibition of sGP binding to neutrophils and GP binding to endothelium is likely to ameliorate the effects of acute Ebola virus infection.

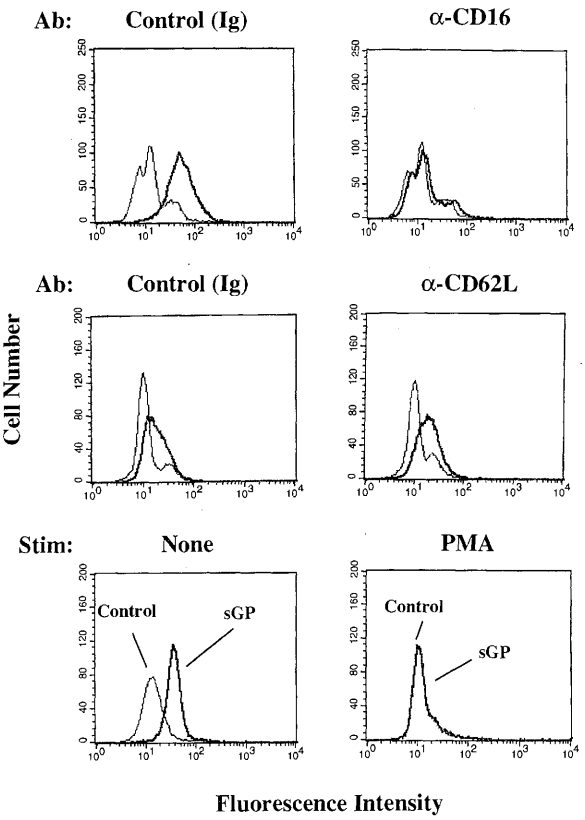

Fig. 3. Dependence of sGP binding on CD16b and correlation of binding with neutrophil activation. Neutrophils were incubated for 30 min at 4°C with a mouse antibody to CD16b (upper panel; clone 3G8 from Immunotech, 1M0813) or CD62L (middle panel, R&D Systems), compared with the indicated control antibody [purified mouse IgG (Vector Laboratories, I-2000], followed by supernatants from control or sGP-transfected 293 cells, primary rabbit antibody to sGP, and a FITC-conjugated secondary antibody to rabbit IgG (Fig. 1, legend). Cells were washed with PBS, fixed in 1% formaldehyde, and analyzed by FACS. For blocking, 10^6 cells were incubated with 0.5 to 1 μg of the relevant antibodies for 30 min in a 50-μl volume. For the lower panel, immunostaining with sGP was performed on isolated neutrophils, which were maintained in media (none) or incubated with PMA (10 ng/ml) at 37°C for 30 min (PMA). Stim, stimulation.

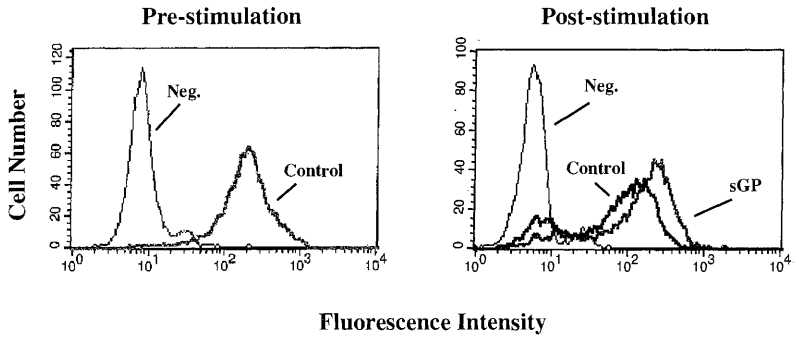

Fig. 4. Effect of sGP on neutrophil function. Exposure of neutrophils to sGP inhibits down-modulation of L-selectin. Isolated neutrophils were incubated with the indicated control- or sGP-containing supernatants (8) and defined media (AIM V, Gibco) for 4 hours at 37°C. Expression of L-selectin was determined with anti-CD62L (R&D Systems), followed by the secondary staining with a FITC-conjugated anti-mouse IgG (Sigma, F2883) as described in Fig. 1. Cells were washed with PBS, fixed with 1% formaldehyde, and analyzed by FACS for relative levels of fluorescence intensity as a function of cell number. An isotype control was used to quantitate background levels of immunostaining (Neg.). Results are representative of three independent experiments.

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15. Recombinant retroviruses were produced by transient transfection of human embryonal kidney 293T cells: 2 × 10^6 cells were plated 24 hours before transfection in 60-mm dishes. Transfection was performed by calcium-phosphate precipitation with 3 μg of a retroviral vector (*14*) encoding luciferase linked to an internal ribosome entry site and a green fluorescent protein derivative (GFP; pEGFP, Clontech), pLZRs-Luc-Gfp; 5 μg of an expression vector encoding gag and pol, pNGVL-MLVgag-pol; and 1 μg of the envelope-encoding plasmid: pNGVL-4070A (ampho) env, pCMV-Eco env, or p1012-Ebola GP, respectively. Supernatants corresponding to 24 to 48 hours after transfection were harvested, cleared by low-speed centrifugation, and either used immediately for infection or frozen at −80°C. Infections were performed in 6-well plates (1.5 × 10^5 to 2.5 × 10^5 adherent cells) or 12-well plates (5 × 10^5 nonadherent cells) with different dilutions of the supernatants by incubating the cells overnight with 1 ml and 300 μl, respectively, of the diluted supernatants. Polybrene was used at a concentration of 5 μg/ml for all the cell lines except for D17, for which the concentration was 100 μg/ml. After overnight infection, fresh medium was added and the cells were incubated for an additional 24 hours. After infection, the cells were lysed in 25 mM tris-phosphate, pH 8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100 and assayed for luciferase activity with Luciferase Assay Reagent (Promega, Madison, WI) in a 1251 BioOrbit Luminometer. The same number of cells (range 5 × 10^4 to 10 × 10^4) was analyzed for every specific cell line.
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The Structure of GABPα/β: An ETS Domain–Ankyrin Repeat Heterodimer Bound to DNA

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GA-binding protein (GABP) is a transcriptional regulator composed of two structurally dissimilar subunits. The α subunit contains a DNA-binding domain that is a member of the ETS family, whereas the β subunit contains a series of ankyrin repeats. The crystal structure of a ternary complex containing a GABPα/β ETS domain–ankyrin repeat heterodimer bound to DNA was determined at 2.15 angstrom resolution. The structure shows how an ETS domain protein can recruit a partner protein using both the ETS domain and a carboxyl-terminal extension and provides a view of an extensive protein-protein interface formed by a set of ankyrin repeats. The structure also reveals how the GABPα ETS domain binds to its core GGA DNA-recognition motif.

Gene expression in eukaryotes is frequently mediated by multiprotein complexes that bind DNA in a sequence-specific manner. This type of transcriptional regulation, termed combinatorial control, is a hallmark of gene regulation in eukaryotic cells. The multiprotein complexes that control eukaryotic gene expression may be composed of structurally similar proteins, such as the Fos and Jun bZIP heterodimer (*1*) or the yeast MATa1/MATα2 homeodomain complex (*2*). In many other cases, genes are regulated by complexes composed of proteins from different structural families. Examples include the complex formed by the MATα2 homeodomain protein with the MCM1 MADS box protein (*3*), and by the herpes simplex VP16 transactivator protein with both the Oct-1 POU-domain protein and host cell factor (*4*). Understanding how transcriptional regulators recruit their partners to form tight, highly specific complexes is central to an understanding of combinatorial control of transcription.

ETS domain proteins make up a large family of DNA-binding proteins found in organisms ranging from fruit flies to humans that play a role in a variety of developmental pathways, in oncogenesis, and in viral gene expression (*5*). These proteins have in common a conserved DNA-binding domain whose structure, as determined for the ETS proteins Fli-1, Ets-1, and PU.1, has an overall topology similar to that of the "winged helix-turn-helix" family of proteins (*6*–*9*). ETS domains bind DNA as monomers and recognize a consensus sequence that contains a core GGA motif. In many cases, greater DNA target specificity is achieved by the cooperative binding of ETS family members with partner proteins (*5*). For example, the related ETS proteins Elk-1, SAP-1, and SAP-2 interact with the serum response factor at the serum response element in the c-Fos promoter (*10*), and the ETS protein PU.1 interacts with Pip on several immunoglobulin light-chain enhancers (*11*).

GA-binding protein (GABP) is a cellular heteromeric DNA-binding protein involved in the activation of nuclear genes encoding mitochondrial proteins (*12*), adenovirus early genes (*13*), and herpes simplex virus immediate-early genes (*14*). The GABP complex is composed of two subunits: an ETS family member, GABPα, and an ankyrin repeat–containing protein, GABPβ (*13*, *15*). Ankyrin repeats, typically 33 amino acids in length, occur in multiple copies in a functionally diverse array of proteins that includes the yeast cell cycle control proteins cdc10/SWI6; the Notch transmembrane protein of *Drosophila melanogaster*; the erythrocyte membrane-associated protein, ankyrin; and IκB, an inhibitor of the transcription factor NF-κB (*16*). GABPβ contains four-and-a-half ankyrin repeats at its NH_2-terminus that mediate heterodimerization with GABPα (*17*). Formation of the GABPα/β heterodimer requires both the GABPα ETS domain and 31 amino acids immediately COOH-terminal to the ETS domain (*17*). The GABPα/β heterodimer binds to DNA sequences containing a core GGA motif with greater affinity than the GABPα subunit alone (*17*, *18*). Two GABPα/β heterodimers associate via the COOH-terminal residues of GABPβ, resulting in a heterotetramer that binds to DNA sequences containing two tandem repeats of the GGA motif (*19*).

To investigate how the structurally dissimilar GABP α and β subunits form a tight heterodimer with enhanced DNA-binding affinity, we determined the crystal structure of the GABPα/β ETS domain–ankyrin repeat heterodimer bound to DNA. Recombinant fragments of mouse GABPα and GABPβ were expressed in *Escherichia coli*, purified as a heterodimer, and crystallized bound to a 21–base pair (bp) DNA fragment (*20*). The structure was solved to 2.15 Å by a combination of multiple isomorphous replacement (MIR) and multiwavelength anomalous dispersion (MAD) methods (Table 1). The model of the GABPα/β- DNA ternary complex presented here contains residues 320 to 429 of GABPα, residues 5 to 157 of GABPβ, and all 21 bp of the DNA.

An overview of the complex (Fig. 1)

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