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DOI
10.1111/j.1348-0421.2001.tb02614.x

Publication date
2001

Published in
Microbiology and Immunology

Link to publication

Citation for published version (APA):
Yamauchi, A., Pötgens, A. J. G., Kuribayashi, F., Nunoi, H., Kanegasaki, S., Roos, D., Malech, H. L., Dinauer, M. C., & Nakamura, M. (2001). Location of the epitope for 7D5, a monoclonal antibody raised against human flavocytochrome b(558), to the extracellular peptide portion of primate gp91(phox). *Microbiology and Immunology*, *45*(3), 249-257. https://doi.org/10.1111/j.1348-0421.2001.tb02614.x

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Microbiol. Immunol., **45**(3), 249–257, 2001

Location of the Epitope for 7D5, a Monoclonal Antibody Raised against Human Flavocytochrome b_{558}, to the Extracellular Peptide Portion of Primate gp91^{phox}

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Received November 30, 2000; in revised form, December 21, 2000. Accepted December 25, 2000

Abstract: **Flavocytochrome b_{558} is the membrane component of the phagocyte NADPH oxidase, and is a heterodimer composed of gp91^{phox} and p22^{phox} subunits. Human flavocytochrome b_{558} is recognized by monoclonal antibody 7D5 at an unidentified extracellular domain, although our previous study suggested it might recognize p22^{phox}. 7D5 has proven useful in rapid screening of individuals for X-linked chronic granulomatous disease by flow-cytometry. Therefore, we re-evaluated the location of the 7D5 epitope using gene-engineered cell lines expressing hybrid flavocytochromes composed of human and murine subunit homologues. The current study demonstrates that the 7D5 recognizes epitope only of primate gp91^{phox}. Flow-cytometric analyses showed that 7D5 consistently bound to cells expressing human gp91^{phox}. In addition, 7D5 immunoprecipitated the ~58 kDa unglycosylated gp91^{phox} protein from solubilized membrane fractions of tunicamycin-treated PLB-985 granulocytes, indicating that glycans were not required for 7D5 binding. Transgenic COS7 cells expressing human gp91^{phox} but not p22^{phox} were recognized by 7D5. These results localized the epitope of 7D5 to an extracellular peptide portion of primate gp91^{phox} and indicate that the antibody will be useful for monitoring the efficiency of gene therapy in patients with flavocytochrome b_{558}-deficient chronic granulomatous disease and for elucidating structural characteristics of flavocytochrome b_{558}.**

Key words: **7D5, Phagocytes, NADPH oxidase, gp91^{phox}**

Flavocytochrome b_{558} is a critical component of the phagocyte NADPH oxidase which generates reactive oxygen intermediates (ROI) essential for microbial killing (6, 22). It is a heterodimeric complex composed of a glycosylated subunit, gp91^{phox} and a non-glycosylated subunit, p22^{phox} (7, 19). It resides in the plasma- and specific granule membranes of phagocytes and B-lymphocytes (21, 25). Primary sequences of both subunits have been determined in humans (20, 23), mice (3, 28), pigs (35, 36), and cows (5), but their membrane topologies have not been resolved with direct experimental evidence. For example, gp91^{phox} has been predicted to have six transmembrane helices based on its primary sequence of 570 amino-acid residues, but the orientation of these helices is uncertain. Gp91^{phox} is the phagocyte oxidase component responsible for transferring electrons from cytoplasmic NADPH to extracellular oxy-

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Abbreviations: CGD, chronic granulomatous disease; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MOPS, morpholinopropanesulfonic acid; PBL, peripheral blood leukocyte; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PVDF, polyvinylidenedifluoride; ROI, reactive oxygen species; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; WBC, white blood cell; X-CGD, X-linked CGD.

gen (6, 22). Therefore, the knowledge of its three-dimensional structure is essential for a better understanding of the molecular basis of electron transfer in the NADPH oxidase. These new structural insights might then also be applied to flavocytochrome homologues in non-phagocytic cells, where signaling by ROI is their primary function rather than microbicidal (1, 10, 12, 27). An attractive approach to elucidate the structure of gp91^{phox} is the development of monoclonal antibodies (mAbs) that bind to the intact polypeptide, because epitopes can be determined by phage display, potentially revealing new structural information about the protein of interest (4, 24).

CYBB, the gene encoding gp91^{phox}, lies on the X chromosome (7, 23, 29). Defects in *CYBB* account for most cases of chronic granulomatous disease (CGD) (65% in U.S.A., 72% in Europe, and 75% in Japan), a disease that is characterized by deficient generation of ROI and life-threatening, recurrent infections (6, 22). X-linked CGD (X-CGD) has been proposed as a suitable target for somatic gene therapy. First, the partial correction of enzyme activity in even a small percentage of phagocytes may be effective; mosaic carriers with 5% or more phenotypically normal phagocytes often have no clinical symptoms (32). Second, X-CGD mice with 5–8% nitrobluetetrazolium-positive peripheral neutrophils after transplantation with syngeneic wild-type bone marrow are resistant to *Aspergillus fumigatus* infection (2). Third, gp91^{phox} gene-transduced X-CGD PLB-985 cells expressing about 25% of normal levels of flavocytochrome b_{558} release normal amounts of superoxide anion upon stimulation by phorbolmyristate acetate (PMA), suggesting that even very inefficient restoration of gp91^{phox} could be effective at increasing phagocyte function (3). Therefore, availability of an established mAb with specificity for the extracellular portion of human gp91^{phox} should be useful for rapid screening of individuals exhibiting clinical characteristics of CGD or to monitor expression of gp91^{phox} in the setting of gene therapy for X-linked CGD.

7D5 is a murine mAb raised against human flavocytochrome b_{558}, and its epitope was previously reported by our laboratory to be located on p22^{phox} using Western blotting (17, 18). In contrast to those findings, the present studies have definitively localized the 7D5 epitope to human gp91^{phox}, the similarity of which can be phylogenetically traced to monkeys but not to animals earlier than primates. Moreover, the antibody binds to unglycosylated gp91^{phox}, indicating that the epitope is directed against a region on the peptide backbone.

Materials and Methods

Antibodies. A mouse mAb 7D5, raised against flavocytochrome b_{558}, was established as described previously (17). A rabbit polyclonal antibody to p22^{phox} and mAb 48 specific for gp91^{phox} were established as described previously (8, 31).

Blood and leukocyte isolation. Blood was drawn into heparinized vacutainer tubes from healthy volunteers after informed consents. Blood of monkeys and mice was withdrawn from artery and heart, under anesthesia with ketamine and diethyl ether, respectively. Blood of non-primate species other than mice was obtained from the Nagasaki slaughterhouse. Human peripheral blood leukocytes (PBLs) were isolated by dextran (1.5%) sedimentation and hypotonic lysis (23 sec under 1/20 isotonicity) either from buffy coats kindly supplied by the Nagasaki Red Cross Blood Center (Nagasaki, Japan) or from whole blood. PBLs of animals were also isolated as above from heparinized blood. Murine peritoneal granulocytes (>90% pure) were collected from peritoneal exudate obtained 16 hr after a 1 ml intraperitoneal injection of 5% starch per each mouse.

Cell lines. Human myeloid leukemia cell line PLB-985 (30) and its gene-transduced derivatives were previously established; these include wild-type PLB-985 (WT PLB), gp91^{phox} knock out PLB-985 (X-CGD PLB) (34), PLB-985 expressing flavocytochrome b_{558} composed of transgenic human gp91^{phox} and endogenous human p22^{phox} (PLB h91/h22) (9), and PLB-985 expressing flavocytochrome b_{558} comprising transgenic murine gp91^{phox} and endogenous human p22^{phox} (PLB m91/h22) (3). Two gene-engineered NIH3T3 cell lines expressing transgenic human gp91^{phox} and endogenous murine p22^{phox} were established by independent methods. Human gp91^{phox} cDNA containing an identical match to the Kozak consensus sequence and partially lacking the 3′-noncoding sequences was cut from pBS-gp91^{phox} Δ3′ at *Bam*HI/*Bam*HI sites and inserted into a *Bam*HI site lying at upstream of internal ribosome entry site sequence of a retroviral vector LZRS-IG (15) resulting in LZRS-gp91^{phox} Δ3′ IG. The vector contains the EBNA-1 gene, *ori*P element of Epstein-Barr virus and puromycin resistance gene for episomal maintenance of the plasmid in virus-producing cells under puromycin selection. The LZRS-gp91^{phox} Δ3′ IG plasmid was transfected into packaging Phoenix-ampho cells by calcium phosphate transfection kit (Life Technologies, LifeTech Orientals, Co., Ltd., Tokyo). After selection by 2 μg/ml puromycin, semi-confluent transfected cells were cultured for one day in puromycin-free medium, which was thereafter filtered and used as a conditioned medium for transduction

of 3T3 cells. 0.5 ml of the conditioned medium was added to 50%-confluent 3T3 cells in a 3-cm diameter 6-well-plate. Green fluorescent protein-positive 3T3 cells were further enriched by 67% using flow-cytometry (FacStar PlusII, Becton-Dickinson, La Jolla, Calif., U.S.A.). In a second approach, 3T3 cells were transduced with MFG, a high-titer retroviral plasmid derived from Molony murine leukemia virus, containing human gp91^{phox} as previously reported, and selected by neomycin (26). Monkey kidney COS7 cell line and a series of its stable transfectants were also used; these included parental COS7 expressing neither endogenous gp91^{phox} nor p22^{phox} (COS7 WT), and COS7 lines expressing one or both of human gp91^{phox} and p22^{phox} transgenes (COS7 gp91, COS7 p22, and COS7 gp91/p22) (33). All cells were cultured in RPMI1640 medium with 2 mM L-glutamine, antibiotics (penicillin, streptomycin, and gentamycin) and 10% fetal calf serum with 5% CO_2 at 37 C. To examine deglycosylated gp91^{phox}, WT PLB, PLB h91/h22, and X-CGD PLB were incubated with or without 2 μg/ml tunicamycin for 3 days in the medium containing a half volume of conditioned medium, namely the supernatant of WT PLB cell suspension cultured for 3 days. More than 60% cells were viable based on trypan blue dye-exclusion test.

Immuno-staining of cells with 7D5 for flow-cytometry. Whole blood (50 μl) or 10^5 cells from transfected cell lines were washed once with phosphate-buffered saline (PBS) and each pre-incubated in 50 μl of PBS containing 10 μg of human immunoglobulins (Cohn Fraction II, III, Sigma Aldrich Japan Co., Ltd., Tokyo) for 10 min to block non-specific binding. The suspended cells were then incubated with purified 7D5 (final concentration 1 μg/50 μl), in the presence (Fig. 2B) or absence of 0.3 μM PMA and 20 μM cytochalasin B, for 1 min at 37 C. Then the cells were incubated for an additional 2 hr at 4 C. Thereafter, the cells were washed twice with staining buffer (PBS containing 0.5% bovine serum albumin and 5 mM ethylenediaminetetraacetic acid. The cell suspension (50 μl) was indirectly labeled with 1 μg of fluorescein isothiocyanate (FITC) or phycoerythrin-conjugated goat-anti-mouse-IgG (Bio Source International, Camarillo, Calif., U.S.A.) by incubation at room temperature for 30 min without (PMA-untreated samples) or with cytochalasin B (PMA-treated samples). Cells were washed once with the washing buffer. Whole blood samples were then hemolyzed by addition of 1 ml of lysing reagent (Ortho Diagnostics, Inc., Raritan, N.J., U.S.A.). Flow-cytometry was performed with FACScan (Becton Dickinson).

Superoxide generation assay. Superoxide generation was monitored by superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction as described previously (16). The difference in absorbance between 550 nm and 540 nm in a 0.4 ml cell suspension (5×10^4 cells) in PBS containing 100 μM ferricytochrome *c* was monitored on a SHIMADZU UV-3000 dual-wavelength spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). The reduced-minus-oxidized difference in millimolar absorption coefficiency of cytochrome *c* between 550 nm and 540 nm was 21 under our conditions. We stimulated the cells with 0.3 μM PMA to generate superoxide anion which subsequently reduced extracellular ferricytochrome *c*. Exogenous SOD (400 U) abolished almost all reduction, indicating that was exclusively dependent on superoxide anion.

Immunoblotting of membrane fractions. We prepared a leukocyte membrane fraction (Fraction C) rich in flavocytochrome b_{558} using the method described by T. G. Gabig (11). The content of flavocytochrome b_{558} of the fraction was calculated from its reduced-minus-oxidized difference spectrum in Tris buffer at 558 nm using the absorption coefficient of 20/mM/cm. The fraction, containing 0.1 pmol of flavocytochrome b_{558} in 50 mM Tris-HCl (pH 8.5) and 3 M urea, was dotted into polyvinylidenedifluoride (PVDF) membrane filter (Immobilon Transfer Membranes, Millipore, Bedford, Mass., U.S.A.) which had been half-dried after being soaked in 100% methanol and subsequently in PBS. The PVDF membrane with dots was then blocked in 5% casein solution for 1 hr and washed once with Tris buffer (0.1 M Tris, 0.1 M NaCl, pH 8.5, containing 0.15% Tween-20). The membrane was then incubated in Tris buffer containing 1 mg/ml of human globulins (Cohn Fraction II, III, Sigma-Aldrich) at room temperature for 10 min to block non-specific binding. The membrane was then incubated over night at 4 C in Tris buffer containing FITC-conjugated 7D5 (final concentration 1 μg/ml), prepared using FITC conjugating kit (Boehringer-Mannheim, Germany) as recommended by the manufacturer. Subsequently the membrane was incubated at 4 C for 1 hr in Tris buffer containing 1,000-fold diluted anti-FITC antibody conjugated with alkaline phosphatase (Pharmacia Biotech, Buckinghamshire, U.K.) as a second antibody, and developed in Tris-HCl (pH 9.5) containing 330 μg/ml nitrobluetetrazolium (Promega, Madison, Wisc., U.S.A.)/165 μg/ml *bis*-chlorophenol (Zymed Laboratories Inc., South San Francisco, Calif., U.S.A.), 50 mM $MgCl_2$ and 20 mM levamisole (Sigma) (15 min, room temperature). Between these steps and after the final incubation, the membrane was washed two times with appropriate buffers.

Immunoprecipitation of deglycosylated gp91^{phox} *and Western blotting.* To perform immunoprecipitation of gp91^{phox}, 7D5 was first conjugated to NHS-activated Sepharose 4B (Amersham Pharmacia Biotech, UK Ltd.)

according to the manufacturer's instructions (7D5-beads). The 7D5-beads were washed and suspended in 25 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 125 mM KCl, 0.1% Triton N101 and then incubated for 4 hr at 4 C with the solubilized membrane which had been obtained from the Fraction C (11) of tunicamycin-treated or -untreated WT PLB cells as reported previously (17). The beads were washed twice with 25 mM MOPS (pH 7.0) containing 1 M NaCl and 0.01% Triton N101. To elute specifically bound antigens from the 7D5-beads, the beads were resuspended in 0.1 M glycine buffer (pH 2.0) containing 0.1% Triton N101, and immediately centrifuged (300×g, 30 sec). Supernatant was neutralized immediately by the addition of one-tenth volume of 2 M $NaHCO_3$ (pH 7.0) containing 2 M NaCl, and then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after treatment with 5 mM dithiothreitol (Novex, San Diego, Calif., U.S.A.) at 70 C for 5 min. After transfer to PVDF membrane, gp91^{phox} and p22^{phox} were probed with mAb 48 and rabbit polyclonal antibody to p22^{phox} followed by horseradish peroxidase-conjugated anti-mouse IgG second antibody. Proteins were visualized using an ECL Western blotting detection kit (Amersham Pharmacia Biotech, UK Ltd.).

Results

7D5 Epitope Is Not Detected in Granulocytes of Animals Phylogenetically Earlier than Primates

To examine the phylogenetic similarity of human flavocytochrome b_{558} with that expressed in other mammals, we stained peripheral blood granulocytes of various origins with 7D5 and analyzed them flow-cytometrically. Human granulocytes and granulocytes from Japanese monkeys exhibited staining with 7D5 (Fig. 1). However, neither murine, porcine, rabbit nor bovine granulocytes were stained with 7D5 (Fig. 1) even after the cells were stimulated with PMA to activate the NADPH oxidase to generate superoxide anions (data not shown). In addition, 7D5 immunocytochemically stained granulocytes of a marmoset, a New World monkey, but not those of horses, dogs, chickens and carps (data not shown). Since these non-primate mammalian granulocytes expressed flavocytochrome b_{558} demonstrable by Western blotting using a polyclonal antibody raised against a carboxyl-terminal peptide of human gp91^{phox} (14) and/or reduced-minus-oxidized absorption spectrum (data not shown), their lack of staining reflected the absence of cross-reaction in 7D5 with flavocytochromes of non-primate mammalian cells. These results demonstrated that the species specificity of the 7D5 epitope included both Old and New World primate species but not animals of earlier phylogeny than primates.

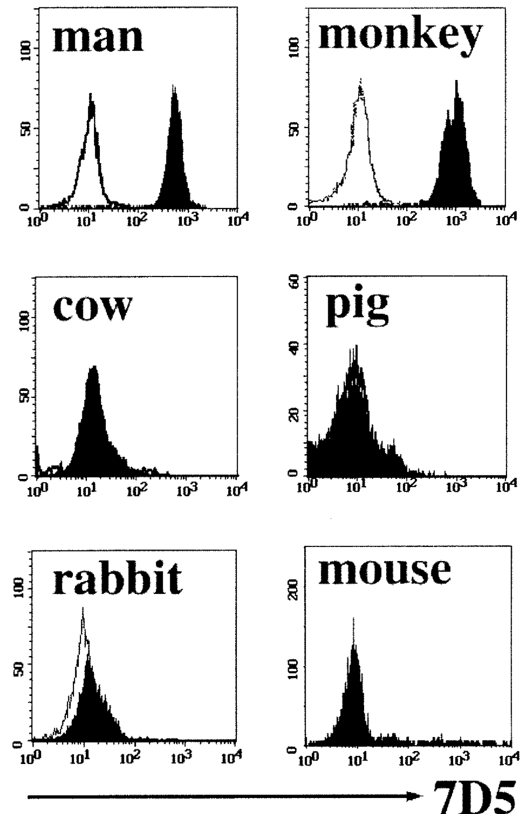

Fig. 1. Flow-cytometric analyses of 7D5-treated granulocytes from various mammals. Granulocytes freshly isolated form several mammalian species were stained with 7D5 as described in "Materials and Methods." Filled and empty histograms indicate samples stained with 7D5 and control IgG$_1$, respectively.

Human gp91^{phox} Is Essential for Expression of the 7D5 Epitope in Flavocytochrome b$_{558}$

To identify which subunit of flavocytochrome b_{558} was essential for binding of 7D5, we analyzed four types of PLB-985 derivatives; WT PLB, X-CGD PLB, PLB h91/h22, and PLB m91/h22. After differentiation with dimethylformamide, WT PLB, PLB h91/h22, and PLB m91/h22 all released superoxide after stimulation with PMA (Fig. 2A). Hence, it can be inferred that these cells significantly express functional flavocytochrome b_{558} on their cell surface due to extracellular release of superoxide. We then examined 7D5 binding to each type of PLB-985 cells by flow-cytometric analysis (Fig. 2B). In contrast to WT PLB and PLB h91/h22 cells, PLB m91/h22 cells were not stained with 7D5 like X-CGD cells, even if these had been stimulated with PMA (Fig. 2B). To rule out the possibility that an intracellular

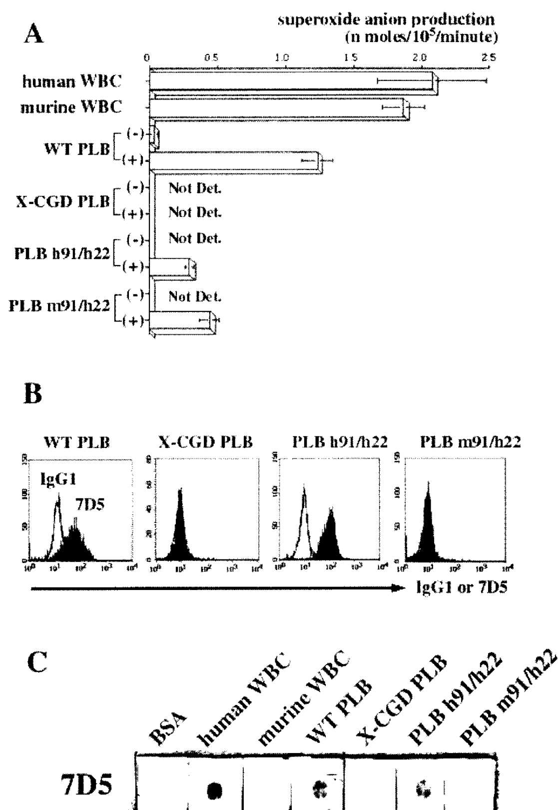

Fig. 2. Determination of the surface expression of h91/h22 or m91/h22 flavocytochrome b_{558} versus wild-type or X-CGD PLB-985 cells using 7D5. A. Superoxide-generating activity was assayed as the SOD-inhibitable reduction of exogenous ferricytochrome *c*. Each bar is the mean of three assays with S.D. (−) refers to cells cultured in medium containing no DMF. (+) refers to cells cultured in medium containing 0.65% DMF for 6 days to be differentiated. Not. Det. = not detectable. B. Flow-cytometry of 7D5 binding to PMA-stimulated PLB-985 cell lines. Four kinds of PLB-985 cells were induced to differentiate with 0.65% DMF and were stained with 7D5 (filled histograms) or control murine IgG_1 (empty histograms) as described in "Materials and Methods." C. Immunostaining of PVDF membranes dotted with membrane fractions from peripheral leukocytes and PLB-985 cells. The dots were stained with FITC-conjugated 7D5 as described in "Materials and Methods." The abbreviations are defined as follows: Human WBC = human peripheral leukocytes; murine WBC = mouse peritoneal exudate leukocytes; WT PLB = wild-type PLB-985; X-CGD PLB = gp91^{phox} knock out PLB-985; PLB h91/h22 = PLB-985 expressing transgenic human gp91^{phox} and endogenous p22^{phox}; PLB m91/h22 = PLB-985 expressing transgenic murine gp91^{phox} and endogenous p22^{phox}; BSA = bovine serum albumin. For further details, see "Materials and Methods."

form of hybrid flavocytochrome b_{558} composed of murine gp91^{phox} and human p22^{phox} has the epitope for 7D5, we dot-blotted membrane fractions of PLB m91/h22 cells and other PLB derivatives on PVDF membrane filter and probed the membrane with 7D5 (Fig. 2C). Although dots of WT PLB and PLB h91/h22 cells were stained with 7D5, the dots of PLB m91/h22 and X-CGD PLB cells were not stained. Therefore, the hybrid flavocytochrome b_{558} comprising murine gp91^{phox} and human p22^{phox} expressed the 7D5 epitope neither at plasma membrane nor in any other subcellular membrane vesicles. These results indicated that gp91^{phox} of human origin was essential for the 7D5 epitope in flavocytochrome b_{558}.

To determine whether or not human [primate] origin p22^{phox} was essential for the expression of the 7D5 epitope in flavocytochrome b_{558}, we analyzed murine NIH3T3 fibroblasts (Fig. 3). Parental NIH3T3 cells (WT NIH3T3) expressed neither endogenous gp91^{phox} (data not shown) nor p22^{phox} (Fig. 3A, lower third column of the lower panel) as reported previously (33) because the cells primarily did not express endogenous gp91^{phox} and therefore murine p22^{phox} alone cannot exist stably in spite of its constitutively active gene. In contrast, human gp91^{phox}, along with endogenous murine p22^{phox}, was definitely present in

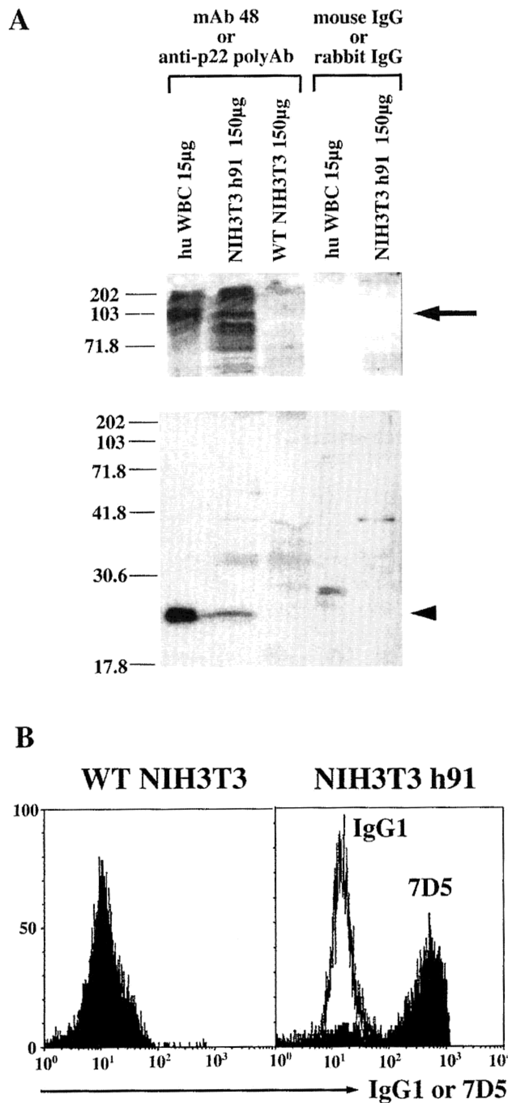

Fig. 3. Expression of hybrid h91/m22 flavocytochrome b_{558} in transgenic NIH3T3 cells and its binding to 7D5. A. Membrane fractions of NIH3T3 cells expressing and not-expressing transgenic human gp91^{phox} (NIH3T3 h91 and WT NIH3T3, respectively) were analyzed by Western blottings (See "Materials and Methods" for more details). Human gp91^{phox} and murine p22^{phox} were detected using a mAb 48 (anti-human gp91^{phox}, upper panel) and a rabbit polyclonal anti-p22^{phox} antibody (lower panel), respectively. Murine IgG$_1$ and non-immune rabbit IgG were used as control antibodies. Human PBLs (hu WBC) were used for positive cells expressing human flavocytochrome b_{558}. An arrow and an arrowhead indicate positions of gp91^{phox} and p22^{phox}, respectively. B. Flow cytometry of wild-type and transgenic NIH3T3 cell lines. Cells were incubated with 7D5 (filled histograms) or nonspecific IgG$_1$ (empty histograms) as the first antibody and PE-conjugated second antibody. Data are shown as in Fig. 2B.

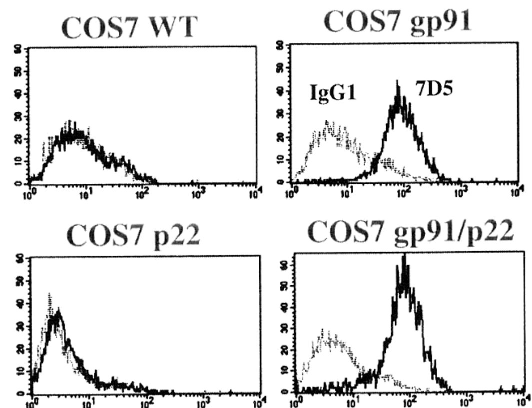

Fig. 4. 7D5 binding to transgenic COS7 cells expressing human gp91^{phox} in the absence of p22^{phox}. COS7 cells transduced with human gp91^{phox} and/or p22^{phox} were stained with 7D5 (solid histograms) and FITC-conjugated second antibody. Murine IgG$_1$ was used as nonspecific antibody (dotted histograms). The abbreviations are as follows: COS7 WT=wild-type COS7; COS7 gp91=COS7 cell line expressing human gp91^{phox} without p22^{phox}; COS7 p22=COS7 cell line expressing human p22^{phox} without gp91^{phox}; COS7 gp91/p22=COS7 cell line expressing both human gp91^{phox} and human p22^{phox}.

membrane extracts of NIH3T3 h91cells that contained a transgene with the human gp91^{phox} cDNA (Fig. 3A, second columns of both panels). The expression of both subunits was, however, low in the extracts comparing to that in the extracts of normal human PBL (Fig. 3A, first columns in both panels). NIH3T3 h91 cells exhibited a significant immunostaining with 7D5 in flow-cytometry in contrast to WT NIH3T3 cells (Fig. 3B). Therefore, we conclude that the epitope for 7D5 is located solely on human gp91^{phox} and suggest that p22^{phox} supports the epitope to be expressed correctly on primate gp91^{phox} in flavocytochrome b_{558}.

7D5 Epitope Does Not Require p22^{phox}

As human gp91^{phox} was co-expressed with its counterpart (p22^{phox}) in normal phagocytes, and transgenic PLB-985 (Fig. 2) and NIH3T3 h91 cells (Fig. 3), it was not clear whether or not a part of p22^{phox} was directly involved in the 7D5 epitope. Therefore we used flow-cytometry (Fig. 4) to analyze previously described COS7 cell lines stably expressing transduced human gp91^{phox} in the absence of endogenous p22^{phox} (33). The results indicated that the cell surface staining of COS7 gp91 with 7D5 (right upper histogram) was similar to that of COS7 gp91/p22 expressing both human gp91^{phox} and human p22^{phox} (right lower histogram). In conjunction with the findings described above, these results indicate that co-expression of p22^{phox} is not required to generate the epi-

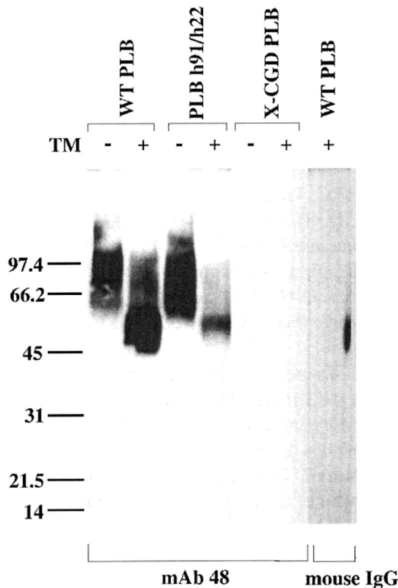

Fig. 5. Western blotting of 7D5 antigens immunoprecipitated from lysed membranes of tunicamycin-treated or untreated PLB-985 cells. Membrane fractions of PLB-985 cells cultured in the presence (+) or absence (−) of tunicamycin (TM) were lysed and incubated with immobilized 7D5 as mentioned in "Materials and Methods." Immunoprecipitates from wild-type and transgenic PLB-985 cell lines (see Fig. 2) were analyzed by Western-blotting as shown in Fig. 3A using only mAb 48 and mouse IgG as primary antibodies. Abbreviations are as follows: WT PLB = wild-type PLB-985; X-CGD PLB = gp91^{phox}-knock out PLB-985; PLB h91/h22 = PLB-985 expressing transgenic human gp91^{phox} and endogenous human p22^{phox}.

tope for 7D5 on human gp91^{phox}, and suggest that the extracellular portion of human gp91^{phox} was necessary and sufficient to form the epitope for 7D5.

Polypeptide Portion of gp91^{phox} *Is Sufficient for 7D5 Binding*

As gp91^{phox} is a glycoprotein, we performed experiments to determine whether the N-linked carbohydrates of gp91^{phox} were required for 7D5 binding. PLB h91/h22 cells were cultured with or without tunicamycin, a fungal metabolite which inhibits the transfer of high-mannose containing carbohydrates from dolichol phosphate onto newly forming polypeptides, and flavocytochrome b_{558} was affinity-purified on 7D5-beads and analyzed by Western blotting using mAb 48. In the sample purified from the tunicamycin-treated cells, most of the broad band of gp91^{phox} around 80–100 kDa is absent and instead contains a new single band having a molecular mass of around 55 kDa (Fig. 5, second and fourth columns), which is the predicted size of the gp91^{phox} core unglycosylated protein. Therefore, we conclude that 7D5 recognizes an extracellular polypeptide region of primate gp91^{phox} and that the epitope is not dependent on carbohydrate.

Discussion

Given the successful application of mAb 7D5 as a tool for the rapid screening of individuals with clinical symptoms of CGD, we have examined in greater detail, the identity and characteristics of the epitope for 7D5. Here we report that gp91^{phox} of primates, but not that of non-primate animals, has the epitope for mAb 7D5. The epitope is expressed on the surface of neutrophils, eosinophils, monocytes/macrophages and B-lymphocytes (13, 14, 17). The primary structure of gp91^{phox} has been deduced from nucleotide sequences in several animal species including humans (3, 5, 20, 23, 28, 35, 36). However, the membrane topology and/or orientation of gp91^{phox} are still incompletely characterized. From tunicamycin-treated PLB h91/h22 cell lysates, 7D5 immunoprecipitated the unglycosylated gp91^{phox} protein. Therefore 7D5 recognizes an extracellular domain shaped by a peptide moiety of gp91^{phox}. Further identification of particular amino acid sequences and/or tertiary structure that are common in primates but different in non-primates and also responsible for 7D5 binding will define an extracellular domain of this protein and presumably articulate flavocytochrome b_{558} structure.

Defining the three-dimensional structure of gp91^{phox} would enhance greatly our understanding of the molecular mechanism of electron transfer from intracellular NADPH to extracellular molecular oxygen and also of its interaction with other subunits in the oxidase complex. The antigen-binding region of an antibody can mimic the three-dimensional conformation of its epitope. Therefore, it should be possible to utilize phage display for the exact identification of the 7D5 epitope as has been done with other flavocytochrome b_{558}-specific antibodies (4, 24).

Neither flavocytochrome b_{558} subunit appears to be stably expressed independently on the surface of phagocytes nor on B-lymphocytes; gp91^{phox} is absent from CGD phagocytes with a primary genetic defect in p22^{phox}, and p22^{phox} is absent from CGD cells with a primary defect in gp91^{phox}. Our flow-cytometry has shown that COS7 gp91 cells which express transgenic human gp91^{phox} as the only component of flavocytochrome b_{558} still exhibited cell surface binding of 7D5, suggesting that the epitope recognized by 7D5 could be maintained even in the absence of p22^{phox}.

Our previous finding of 7D5 epitope on p22^{phox} (18) was based on the data of Western blotting. It was how-

ever not reproducible. The data is consistent with present results only when fragmented gp91^{phox} containing the epitope for 7D5 is partially renatured in detergent-containing buffer. We tried but failed to reproduce previous data using various renaturation procedures. Dots that can be immunostained with 7D5 as illustrated in Fig. 2C lost their immunogenicity to 7D5 after treatment with ethanol to deplete lipids, suggesting 7D5 epitope required a strict conformation supported by lipids (data not shown). As the antigenicity of the dots was resistant to 3 M urea (Fig. 2C), lipids but not hydrogen bindings is crucial for keeping 7D5 epitope intact.

As far as we know, 7D5 is the only antibody that recognizes an external epitope of flavocytochrome b_{558}. It has been used for the rapid diagnosis of X-linked CGD and for the detection of X-linked CGD cells corrected by gene therapy. Localizing the epitope to gp91^{phox} has further established reliability for its use in the diagnosis and monitoring of gene therapy of X-linked CGD.

We greatly thank Dr. William M. Nauseef and Dr. Frank R. DeLeo, Division of Infectious Diseases, Veterans Administration Medical Center and University of Iowa, for helpful discussion in manuscript preparation.

This work was supported by Grants-in-Aids of The Japanese Ministry of Education, Science, Sports, and Culture, Cooperation Research Grants of Institute of Tropical Medicine, and Grants for COE of Japan (to Nakamura, M.), and by NIH ROLHL45635 (to Dinauer, M.C.).

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