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Production and characterization of a monoclonal antibody to a human interferon-induced double-stranded RNA-binding M_r 68,000 protein kinase

(immunoblotting/immunoprecipitation/immunoaffinity chromatography)

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ABSTRACT **One of the interferon-induced proteins thought to be involved in the antiviral effects of interferon is a double-stranded RNA-dependent protein kinase. This paper reports the development of a monoclonal antibody, 10A5, that recognizes a protein that co-migrates with the double-stranded RNA-dependent protein kinase at an approximate molecular weight of 68,000. Levels of this protein and of the protein kinase activity increase 3-fold on interferon treatment of T98G cells. The specificity of the monoclonal antibody was determined by ELISA, immunoblotting, and immunoprecipitation procedures. Furthermore, immunoaffinity chromatography of an interferon-induced T98G cell extract previously phosphorylated in the presence of double-stranded RNA and radiolabeled ATP resulted in the specific elution of a phosphorylated M_r 68,000 protein from the monoclonal antibody 10A5-Sepharose column. Monoclonal antibody 10A5 recognizes both native and denatured protein kinase, independent of double-stranded RNA binding or phosphorylation, and should therefore serve as a useful tool in analyzing the role of the double-stranded RNA-dependent protein kinase in the mechanism of interferon action.**

The interferons (IFNs) are a family of proteins that exert many varied effects on a wide range of responsive cells. The IFNs inhibit virus replication, regulate cell growth, and modulate the immune response. A diverse number of biochemical responses can also be effected, probably through the induction of specific cellular genes. A number of new proteins, and increased amounts of others, can be detected in cells following IFN treatment. Although these proteins have been characterized (1–5), their functions have not yet been determined. Two IFN-induced double-stranded (ds) RNA-dependent enzymes have been well characterized, a protein kinase and 2′,5′-oligoadenylate synthetase, both of which initiate pathways leading to inhibition of protein synthesis (reviewed in ref. 6). In human cell-free systems, the ds RNA-dependent protein kinase phosphorylates an endogenous M_r 68,000 protein and the α subunit of the eukaryotic protein synthesis initiation factor eIF2. The enzyme 2–5A synthetase is also activated by ds RNA and generates 2′,5′-linked oligoadenylates from ATP. These in turn activate an endonuclease that cleaves single-stranded RNA. Accumulating evidence (reviewed in ref. 7) shows these two enzymes are involved in the development of the antiviral state induced by IFN. Correlation of the antiviral state with any of the other IFN-induced proteins is less certain.

Monoclonal antibodies (mAbs) specific to IFN-induced ds RNA-binding proteins have been isolated to investigate the role each of the IFN-induced proteins plays in mediating the effects of IFN. In this paper, we report the derivation of mAb directed against one of these proteins. This protein (M_r, 68,000) increases in amount in response to IFN treatment and is phosphorylated by ds RNA-dependent protein kinase. Whether the ds RNA-dependent phosphorylation of the M_r 68,000 protein represents an autophosphorylation of this protein kinase or the phosphorylation of a M_r 68,000 substrate protein has yet to be resolved. For simplicity, this system is referred to as M_r 68,000 kinase (8). The specificity of mAb 10A5 (IgM) to the M_r 68,000 kinase was determined by an ELISA and by immunoblotting, immunoprecipitation, and immunoaffinity chromatography. This mAb should prove useful in characterization of the M_r 68,000 kinase system and for further investigation of the role this system plays in the mechanism of action of interferon.

MATERIALS AND METHODS

Characterization of IFN-Induced Poly(rI·rC)-Binding Proteins. Confluent T98G (a human glioblastoma cell line, ref. 9) cells grown in 75-cm^2 flasks (Nunc) were incubated with and without human (Hu) IFN-α lymphoblastoid (Ly) (Wellferon, 2×10^8 units/mg; ref. 10) at 200 units/ml in α minimal essential medium (minus methionine)/10% fetal calf serum (BDH) for 4 hr at 37°C in 5% CO_2/95% air. Then, [^{35}S]methionine (New England Nuclear; >800 Ci/mmol; 1 Ci = 37 GBq) was added at 10 μCi/ml, and the cells were incubated for an additional 16 hr, trypsinized, washed, and stored as a pellet at −70°C. The cell pellet was lysed with Nonidet P-40 buffer containing protease inhibitors (11), and the extract (5×10^6 cpm) was added to 60 μl of a suspension of poly(rI·rC)-cellulose (11) equilibrated with cellulose buffer [10 mM Hepes, pH 7.5/90 mM KCl/1.5 mM $Mg(OAc)_2$/7 mM mercaptoethanol/20% (vol/vol) glycerol] for 1 hr at room temperature. The poly(rI·rC)-cellulose suspension was washed three times with cellulose buffer, pelleted, and boiled in Laemmli sample buffer (12), and the supernatants (2×10^4 cpm) analyzed by 10% $NaDodSO_4$/PAGE followed by fluorography using Autofluor (National Diagnostics, Somerville, NJ) and autoradiography.

Assay of ds RNA-Dependent Protein Kinase Activity. *Solution assay.* The procedure of Hovanessian and Kerr (13) was used, with reaction mixtures (10 μl) containing 3–6 μl of cell extract.

Poly(rI·rC)-cellulose assay. A slurry (25 μl) of poly(rI·rC)-cellulose previously washed in cellulose buffer was added to the sample of cell extract to be assayed. The mixture was incubated at 4°C for 1 hr, the poly(rI·rC)-cellulose was washed twice in 10 volumes of cellulose buffer, and the pellet was suspended in 15 μl of cell lysis buffer (11) containing 10

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Abbreviations: mAb, monoclonal antibody; IFN, interferon; ds, double-stranded; RvsMu Ig, rabbit anti-mouse Ig.

mM $Mg(OAc)_2$/100 μM ATP and 1.5 μCi of [γ-^{32}P]ATP (ICN; >7000 Ci/mmol). The mixture was incubated for 30 min at 30°C, and the phosphorylated proteins were resolved by PAGE and autoradiographed. To quantitate the level of phosphorylation in each sample, the area on the autoradiograph specific to the M_r 68,000 protein was cut from the dried polyacrylamide gel and the radioactivity was determined by scintillation counting.

Antigen Preparation. A partially purified extract of IFN-induced poly(rI·rC)-binding proteins was prepared from Wellferon-treated (200 units/ml for 20 hr) T98G cells. The Nonidet P-40 lysate (150 mg) was first passed over a 15-ml F3G4 cibacron blue agarose (Pierce) column, previously washed with HBG buffer [20 mM Hepes, pH 7.5/5 mM $Mg(OAc)_2$/10% (vol/vol) glycerol, 1 mM dithiothreitol] containing 10 mM KCl and the bound proteins were eluted using 3 bed volumes of HBG buffer containing 500 mM KCl. The eluate was immediately diluted to a salt concentration of 100-mM KCl and passed over 4 ml of poly(rI·rC)-agarose (P-L Biochemicals). The column was washed with HBG buffer containing 90 mM KCl, and the IFN-induced poly(rI·rC)-binding proteins were eluted with glycerol-free HBG buffer containing 500 mM KCl. The eluate was diluted to 100 mM KCl, poly(rI·rC) (P-L Biochemical) was added to a final concentration of 1 μg/ml, and the protein preparation was dialyzed overnight against 20 mM Hepes (pH 7.5) and lyophilized. Both IFN-induced protein kinase and 2–5A synthetase (14, 15) activity were retained.

Production of mAbs. Female BALB/c mice were immunized by i.p. injection of 100 μg of the IFN-induced poly(rI·rC)-binding protein preparation in complete Freund's adjuvant. Repeat injections were given in incomplete Freund's adjuvant 14 days later. After a further 11 days, 100 μg of antigen was injected i.v. in phosphate-buffered saline (P_i/NaCl). Splenocytes (1.5×10^8) were isolated 3 days later and fused to 2×10^7 mouse myeloma P3-NSI cells using polyethylene glycol (16). The cell suspension was dispensed into 96-well microtiter plates at a myeloma cell density of 1×10^5 cells per ml in Iscove's modified Dulbecco's medium containing 15% fetal calf serum and hypoxanthine/aminopterin/thymidine selection medium (16). After screening by ELISA, immunoblotting, and immunoprecipitation techniques (see below), hybridoma cells that secreted antibody demonstrating increased specificity to IFN-induced proteins were cloned by limiting dilution. Cells (3×10^6) were injected i.p. into pristane (Aldrich)-primed 4-month-old female BALB/c mice for production of ascitic fluid. Determination of mAb class specificity and quantitation of mAbs in ascitic fluid were both by ELISA.

ELISA for mAbs Directed Against IFN-Induced Proteins. IFN-induced and control antigen preparations (0.1–1 μg/100 μl 0.1 M $NaHCO_2$, pH 8.3, per well) were incubated for 18 hr at 4°C in 96-well plates (Immunoplates, Nunc). The wells were then incubated with bovine serum albumin (Sigma) at 0.2 mg/ml at room temperature for 4 hr, washed, incubated at room temperature overnight with 100 μl of hybridoma culture supernatant, and washed again. Bound antibody was detected by addition of 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgG and IgM antibody (Tago, Burlingame, CA) to each well for 2.5 hr followed by treatment with disodium *p*-nitrophenylphosphate (Sigma). The optical density was read at 410 nm. Antigen, antibody, conjugate, and substrate controls were included in the assay.

Immunoblotting Procedure. Proteins extracted with cell lysis buffer (11) from two human cell lines (T98G and HeLa cells) incubated with or without HuIFN-αLy and from one mouse cell line (L929 cells) treated as control or with HuIFN-$\alpha_2\alpha_1$ (kindly provided by Schering) were resolved by 7.5% PAGE (10 or 60 μg per lane) and transferred to nitrocellulose paper (Schleicher & Schuell) as described (17). The nitrocellulose was incubated with "blotto" solution (5% skim milk powder/P_i/NaCl) (18) for 1.5 hr at 37°C followed by mAb 10A5-specific hybridoma cell ascites fluid (100 μg/ml) for 2 hr at room temperature and then washed five times for 15 min each. Finally, the nitrocellulose was treated with rabbit anti-mouse IgM (RvsMu Ig; Bionetics, Charleston, SC) (4 μg/ml) for 2 hr at room temperature and washed. The specificity of the mAbs was determined by incubation with ^{125}I-labeled protein A (New England Nuclear; 70–100 μCi/μg) followed by extensive washing of the nitrocellulose strips to remove unbound radiolabel and autoradiography. Blotto solution was used in all incubation and washing steps throughout the immunoblotting procedure. Each immunoblotting assay included silver staining (19) and amido black staining (18) of the transferred gel and nitrocellulose, respectively, to assess protein transfer.

Immunoprecipitation Procedure. IFN-induced and control T98G cell extracts labeled with [^{35}S]methionine (1.1×10^7 cpm) were incubated for 2 hr at 4°C with 100 μl of a 10% slurry of *Staphylococcus aureus* cells (IgGsorb, The Enzyme Center, Malden, MA) freshly coupled with RvsMu Ig (IgGsorb-RvsMu Ig) at a 5:1 ratio as described (20). The paired extracts were cleared of nonspecifically bound radiolabeled material and treated overnight at 4°C with 200 μl of a specific hybridoma culture supernatant concentrated to 1/10th vol by ultrafiltration (Amicon). IgGsorb was freshly coupled (20) with RvsMu Ig or with normal rabbit serum, and 50 μl of a 10% slurry of each was incubated with half of the antigen/mAb mixture for 2 hr at 4°C with mixing. The pellets were washed extensively, and the supernatants (10^4 cpm) were analyzed by 10% PAGE and fluorography. The autoradiographs were quantitated by densitometry.

Preparation of the mAb 10A5 Affinity Column. Ascitic fluid produced by hybridoma 10A5 from BALB/c mice was diluted in 0.1 M $NaHCO_3$, pH 8.6/0.5 M NaCl to a concentration of 1 mg of total protein/ml (0.56 mg of mAb/ml) and dialyzed against the same buffer for 24 hr without loss of mAb activity. The protein in this solution was bound to CNBr-activated Sepharose (Pharmacia) at a concentration of 2 mg of protein/ml of Sepharose, which had been washed with the eluting buffer (0.2 M glycine, pH 2.2) and then with binding buffer (0.1 M Tris·HCl, pH 8.0) and stored at 4°C in binding buffer containing 0.02% NaN_3. To phosphorylate the M_r 68,000 protein before carrying out the immunoaffinity chromatography procedure, an IFN-induced T98G cell extract was incubated with radiolabeled ATP and poly(rI·rC) under the conditions of the solution assay (13). This "prephosphorylated" extract was mixed with 10A5-Sepharose (1 ml) at 4°C for 4 hr in a batchwise procedure, and the 10A5-Sepharose was packed into a 3-ml column at a flow rate of 2 ml/min. The column was washed with 20 ml of binding buffer and the bound proteins were eluted with 2 ml of 0.2 M glycine, pH 5.0, collected as 0.5-ml fractions into 100 μl of 1 M Tris·HCl, pH 8.0, and then with 2 ml of 0.2 M glycine, pH 2.2, collected as 0.5-ml fractions into 200 μl of 1 M Tris·HCl, pH 8.0, to neutralize the acidic conditions of the elution buffers. The column fractions were analyzed by 10% PAGE. A control mAb affinity column was prepared using ascitic fluid produced from a hybridoma cell secreting an IgM mAb of different antigenic specificity and chromatographed under the conditions described above. All chromatography was carried out at 4°C.

RESULTS

Characterization of the Poly(rI·rC)-Binding Proteins Induced in T98G Cells by IFN. Treatment of T98G cells with lymphoblastoid IFN results in elevated synthesis of six to eight induced proteins. These proteins are clearly visualized when [^{35}S]methionine-labeled poly(rI·rC)-cellulose-binding

proteins from the IFN-treated cell extracts are examined using PAGE. Six of these IFN-induced proteins (M_r 120,000, 104,000, 98,000, 88,000, 80,000, and 69,000) are shown in Fig. 1*a*. Two proteins (M_r 56,000 and 43,000) are evident in other poly(rI·rC)-binding experiments (data not shown). Phosphorylation of the M_r 68,000 protein was elevated in T98G cells after approximately 4 hr of IFN treatment (Fig. 1*b*, lane 3) while IFN induction and increased phosphorylation of the protein was inhibited by simultaneous treatment with actinomycin D (Fig. 1*b*, lane 9). Untreated T98G cells possess a high level of protein kinase activity, as monitored by phosphorylation of the M_r 68,000 protein, and demonstrate a 3-fold increase in enzyme activity with 24 hr of incubation with IFN (Fig. 1*b*, lanes 1–8).

Specificity of mAb 10A5 to an IFN-Induced Protein. To compare the specificity of mAb binding to antigen preparations produced from IFN-treated and control T98G cell extracts, hybridoma culture supernatants were initially screened by an ELISA. One mAb, 10A5 (IgM), was selected on the basis of the ELISA results for further characterization by immunoblotting. The intensity of mAb 10A5 binding to a specific protein in the IFN-treated preparation was compared with that of the control preparation. Binding of mAb 10A5 to IFN-induced and control proteins immobilized on nitrocellulose was detected by incubation with RvsMu IgM followed by incubation with ^{125}I-labeled protein A and autoradiography. The mAb 10A5 specifically bound to a protein of M_r 67,000–68,000, which was found at elevated levels in the IFN-treated human, but not mouse, cell protein extracts (Fig. 2).

To determine which, if any, of the proteins in their native state would be specifically bound by mAb 10A5, immunoprecipitation experiments were carried out with [^{35}S]methionine-labeled IFN-induced and control T98G cell extracts. The mAb 10A5 precipitated a single protein of M_r 67,000–68,000 (Fig. 3). The level of this protein in the IFN-treated cell extract was twice that in the control cell extract (Fig. 3, lanes 2 and 4). Furthermore, the specificity of this immunoprecipitation by mAb 10A5 is demonstrated by the lack of a precipitable M_r 68,000 protein band when

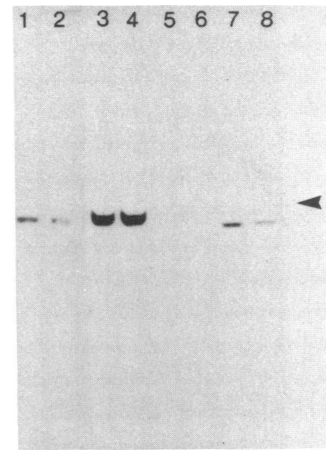

FIG. 2. Immunoblotting of human ds RNA-dependent protein kinase with mAb 10A5. Total cell extracts (10 μg per lane for lanes 1 and 2; 60 μg per lane for lanes 3–8) from IFN-induced (lanes 1, 3, 5, and 7) and control (lanes 2, 4, 6, and 8) T98G cells (lanes 1–4), L929 cells (lanes 5 and 6), and HeLa cells (lanes 7 and 8) were resolved by 7.5% PAGE. The gels were transferred onto nitrocellulose and treated with mAb 10A5. Antigen–mAb complexes were visualized after treatment with RvsMu Ig and ^{125}I-labeled protein A. Migration of the M_r 69,000 marker protein is indicated by the arrowhead.

IgGsorb–normal rabbit serum was used to precipitate antigen–mAb 10A5 complexes or when mAb (IgM) of different specificity was used for the immunoprecipitation (Fig. 3, compare lanes 2 and 4 with lanes 3 and 5–9).

Specific Binding and Elution of the IFN-Induced M_r 68,000 Protein from a mAb 10A5 Affinity Column. Immunoblotting and immunoprecipitation experiments with mAb 10A5 indicated specificity to an IFN-induced protein of M_r 67,000–68,000. The only previously reported protein induced by IFN with that approximate M_r is ds RNA-dependent protein kinase (21). Therefore, to determine whether mAb 10A5 was recognizing this enzyme, ascitic fluid from mice injected with hybridoma cells secreting mAb 10A5 was bound

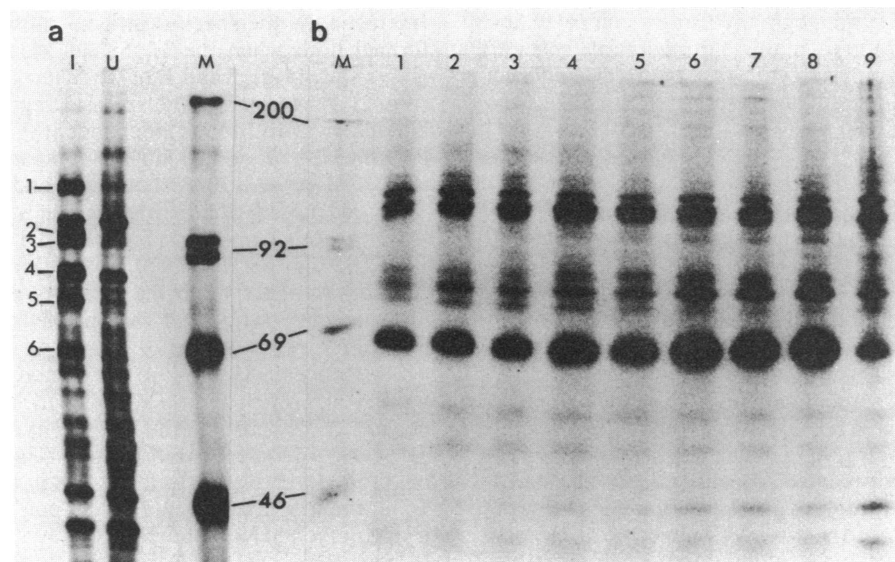

FIG. 1. (*a*) Detection of ds RNA-binding proteins in T98G cells. [^{35}S]Methionine-labeled proteins from IFN-induced (lane I) and control (lane U) human T98G cells were bound to poly(rI·rC)-cellulose, the column was washed, and the proteins were eluted in Laemmli sample buffer. The proteins were resolved by 10% PAGE, which was followed by fluorography and autoradiography. (*b*) Assay of ds RNA-dependent protein kinase activity in human T98G cells. Lanes 1–8: T98G cells treated with Wellferon (200 units/ml) for 0, 2, 4, 8, 12, 16, 20, and 24 hr were assayed by the poly(rI·rC)-cellulose protein kinase assay and phosphorylation of the M_r 68,000 protein detected by 10% PAGE. Lane 9: T98G cells treated simultaneously with Wellferon and actinomycin D (2 μg/ml) for 8 hr were also assayed by the protein kinase assay. Lanes M: protein standard markers ($M_r \times 10^{-3}$).

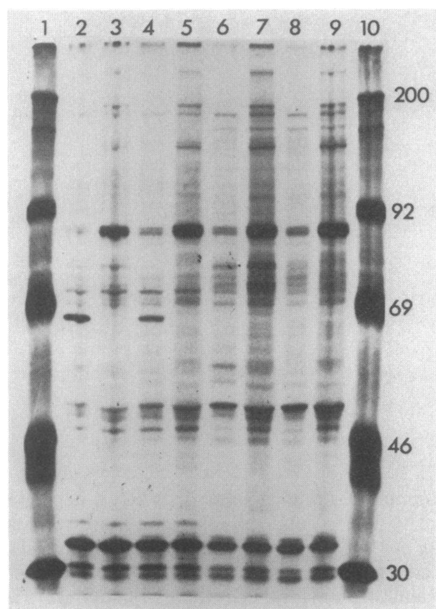

FIG. 3. Immunoprecipitation of [^{35}S]methionine-labeled proteins from human T98G cells with mAb 10A5. T98G cells treated with IFN (lanes 2, 3, 6, and 7) and control cells (lanes 4, 5, 8, and 9) were incubated with mAb 10A5 (lanes 2–5) or with a control mAb (IgM) (lanes 6–9). The antigen–mAb complex was precipitated with IgGsorb–RvsMu Ig (lanes 2, 4, 6, and 8) or with IgGsorb–normal rabbit serum (lanes 3, 5, 7, and 9). Lanes 1 and 10: M_r markers (× 10^{-3}).

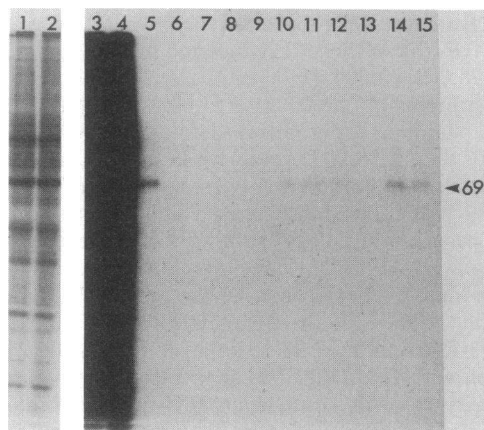

FIG. 4. Chromatography of ^{32}P phosphorylated protein from IFN-treated T98G cells on mAb 10A5-Sepharose. Fractions were analyzed by 10% PAGE. Lanes: 3, 2 μl of total cell extract; 4–7, 20-μl aliquots of 1-ml wash buffer fractions 1, 10, 15, and 20; 8–11, 20-μl aliquots of 0.5-ml, 0.2 M glycine (pH 5.0) elution buffer fractions; 12–15, 20-μl aliquots of 0.2 M glycine (pH 2.2) elution buffer fractions. Lanes 3 and 4 represent longer exposure times of lanes 1 and 2. The arrowhead indicates the position of bovine serum albumin (M_r 69,000) migration.

to CNBr-activated Sepharose for affinity chromatography. The ascitic fluid was titrated against purified IgM in an ELISA and found to contain 17.3 mg of IgM/ml at a purity of 56% total protein. To provide convenient detection of the M_r 68,000 kinase throughout the immunoaffinity chromatography, IFN-treated T98G extract was incubated with [γ-^{32}P]ATP and poly(rI·rC) to allow ds RNA-dependent phosphorylation of the M_r 68,000 protein. The primary protein band that eluted specifically from the mAb 10A5-Sepharose column was resolved by PAGE at the same M_r as the ds RNA-dependent phosphorylating protein (Fig. 4). In contrast, immunoaffinity chromatography using a control mAb-Sepharose column resulted in the elution of no specific phosphoproteins (data not shown). This is direct evidence that mAb 10A5 is directed against the IFN-induced ds RNA-dependent M_r 68,000 kinase.

DISCUSSION

Most cellular proteins that are induced to elevated levels following IFN treatment of the cells (refs. 1–5 and Fig. 1*a*) have not been assigned a specific function, although they may be involved in the overall biochemistry of the IFN system. The two best-characterized enzymes induced by IFN, 2–5A synthetase and ds RNA-dependent protein kinase, can be detected by only their respective activity assays. Although the biochemical pathways involving these enzymes are well understood, the role they play in the mechanism of action of IFNs, particularly in virus-infected cells, is not yet clearly defined. To develop new tools to gain better understanding of IFN-induced proteins, mAbs were developed to a protein preparation from IFN-induced human T98G cells. In this paper, mAb 10A5, which binds specifically to the IFN-induced M_r 68,000 kinase is described.

The level of this M_r 68,000 protein becomes elevated in T98G cells following IFN treatment, and the kinetics, fold increase, and sensitivities to transcriptional inhibitors are similar to those previously reported for the M_r 68,000 kinase of HeLa cells (ref. 21 and Fig. 1*b*). In HeLa cells, ds RNA-dependent protein kinase phosphorylates an endogenous protein of M_r 69,000–72,000 (21) and, by the phosphorylation assays, the enzyme of T98G cells also uses a substrate protein of M_r 69,000–72,000. However, detection of this IFN-induced protein with mAb 10A5 has revealed a shift in gel mobility of the T98G cell substrate protein that may correlate with its state of phosphorylation (22). The antigen recognized by mAb 10A5 migrates at M_r 67,000–68,000 when phosphorylation of the protein has not occurred throughout the procedure (Figs. 1*a*, 2, and 3). The antigen (assumed to be the same because of the monoclonality of the antibody) bound by mAb 10A5 migrates at a slightly higher M_r (69,000–72,000) when phosphorylation of the protein has taken place either before or after mAb–antigen complexing (Figs. 1*b* and 4).

The antigenic epitope bound by mAb 10A5 is unclear. Interestingly, the specific antigen–antibody complex can be formed both when the M_r 68,000 kinase antigen is in the native state (Figs. 3 and 4) or when it is in the denatured state (Fig. 2). Furthermore, the antigenic epitope recognition and binding by mAb 10A5 is independent of ds RNA having been bound to the M_r 68,000 kinase (compare Fig. 4 with Figs. 2 and 3). Also, mAb 10A5 recognizes and binds to the specific M_r 68,000 protein whether the antigen is phosphorylated (Fig. 4) or nonphosphorylated (Figs. 2 and 3). The ability of mAb 10A5 to bind the M_r 68,000 kinase throughout these varied conditions suggests that the antigenic determinant it recognizes may be "sequential" as opposed to "conformational" in nature.

The antigenic epitope of the human M_r 68,000 substrate protein of protein kinase recognized by mAb 10A5 is not present or is masked in the equivalent mouse protein kinase M_r 64,000–67,000 substrate protein. The mAb 10A5 does not bind to the mouse substrate protein in immunoblotting (Fig. 3) or immunoprecipitation (data not shown) procedures. Using polyclonal antibodies to the human M_r 68,000 kinase, Laurent *et al.* (8) recently found a similar lack of cross-reactivity to the mouse M_r 64,000–67,000 substrate protein of ds RNA-dependent protein kinase. These results further support that differences exist in the polypeptide structures of these phosphoproteins (22). Since mAb 10A5 recognizes both native and denatured protein, independent of ds RNA binding or phosphorylation, it should aid detailed biochemical analysis of the M_r 68,000 kinase by offering a means of obtaining

homogeneous and active protein. It remains uncertain whether the M_r 68,000 protein is a substrate of protein kinase or the enzyme itself (23). With active immunoaffinity-purified M_r 68,000 protein, its relationship to the protein kinase can be ascertained. Furthermore, *in situ* immunofluorescence studies using the mAb may ultimately determine the involvement of this kinase in the antiviral action of IFN.

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