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# MECHANISM OF INTERFERON ACTION
## Production and Characterization of Monoclonal and Polyclonal Antibodies to the Interferon-induced Phosphoprotein $P_1$

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SUMMARY: Monoclonal and polyclonal antibodies to the interferon-induced phosphoprotein $P_1$ were prepared using protein $P_1$ purified from human amnion U cells as the immunogen. Rabbit antiserum to protein $P_1$ recognized with comparable efficiency $P_1$ both from human U cells and from mouse L929 cells. Immunoprecipitates that contained protein $P_1$ also possessed a protein kinase activity that catalyzed the phosphorylation of protein $P_1$ and the $\alpha$ subunit of initiation factor eIF-2. Three BALB/C mouse monoclonal antibodies efficiently recognized human protein $P_1$, but either did not recognize or recognized very poorly $P_1$ from mouse cells. A fourth monoclonal antibody against human $P_1$ recognized mouse $P_1$ with nearly equal efficiency. Immunoprecipitation of human $P_1$ with different sequential combinations of the monoclonal antibodies suggest that two antigenic classes of protein $P_1$ may exist. 

Protein phosphorylation-dephosphorylation may play an important role in the mechanism of the antiviral action of IFN (1,2). Treatment of animal cells with IFN results in the induction of a double-stranded RNA (dsRNA) -dependent protein kinase which catalyzes the phosphorylation of two proteins, $P_1$ and $\alpha$. Protein $P_1$ is a ribosome-associated protein of Mr 64,000-70,000, depending on the species of cell examined and the state of phosphorylation (3-5). $\alpha$ is the smallest subunit of protein synthesis initiation factor eIF-2 (6). The translation of viral mRNA is greatly inhibited in Type I IFN-treated murine (7) and human (8) cells. This translational control may occur as the result of induction of the $P_1$/eIF-2$\alpha$ protein kinase in IFN-treated cells and subsequent phosphorylation of eIF-$\alpha$ (9). Phosphorylation of $\alpha$ is believed to affect the efficiency of cycling of eIF-2 during the initiation of protein synthesis (10), and provides an important mechanism of translational control (11).

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Four lines of evidence suggest that the IFN-induced $P_1$/eIF-2α protein kinase activity resides in protein $P_1$. First, the $P_1$/eIF-2α protein kinase activity copurifies with protein $P_1$ (5). Second, the apparent native molecular weight of the $P_1$/eIF-2α protein kinase (5) is comparable to the molecular weight of denatured phosphorylated protein $P_1$ (4). Third, $P_1$ phosphorylation reaction kinetics are independent of reaction volume, which suggests that the phosphorylation of $P_1$ may be an intramolecular autophosphorylation (5). Fourth, protein $P_1$ possesses a dsRNA-dependent nucleotide binding site as established by photoaffinity labelling with ATP (12). As part of our studies of the $P_1$/eIF-2α protein kinase induced by IFN, we report in this communication the production and partial characterization of monoclonal and polyclonal antibodies to the IFN-induced phosphoprotein $P_1$ purified from IFN-treated human amnion U cells.

## MATERIALS AND METHODS

Materials. Hypoxanthine and thymidine were from Calbiochem. Aminopterin was from ICN. Protein A-Sepharose was from Sigma. A mouse monoclonal antibody isotype kit from American Qualex was used for determining immunoglobulin subtype. P3X63-Ag8.653 myeloma cells were obtained from Dr. R. J. Scibienski, University of California, Davis, CA. The sources of all other materials and reagents have been described previously (5,14).

Cells and Interferon. The growth of human amnion U cells and mouse fibroblast $L_{929}$ cells were as previously described (3,5,9,12). Sendai virus-induced human leukocyte IFN was generously provided by Dr. Kari Cantell, Central Public Health Laboratory, Helsinki, Finland; the specific activity was $1.9 \times 10^6$ units per mg protein. Natural mouse IFN induced in $L_{929}$ cells by the Herts strain of Newcastle disease virus was prepared as previously described (13); the specific activity was about $10^5$ units per mg protein.

Kinase Purification. Purification of the dsRNA-dependent protein kinase from groups of 12 to 18 confluent roller bottle cultures of IFN-treated human amnion U cells was as described in detail elsewhere (14). For immunizations, purification through the DEAE cellulose chromatography step was utilized followed by preparative $NaDodSO_4$ polyacrylamide gel electrophoresis (14).

$P_1$-specific Antiserum. Rabbit antiserum to protein $P_1$ from human U cells was prepared by subcutaneous and intramuscular immunization of a New Zealand albino rabbit with polypeptide $P_1$-containing $NaDodSO_4$-polyacrylamide gel slices which had been emulsified in Freund's complete adjuvent (15). One booster immunization in incomplete Freund's adjuvent and two booster immunizations of antigen alone were given at three-week intervals. Polyclonal antibodies to $P_1$ were then purified from the immune rabbit serum by protein A-Sepharose chromatography. Immunoglobulins were eluted from protein A-Sepharose with 0.1 M glycine at pH 4.0.

$P_1$-specific Hybridomas. Four female BALB/c mice were immunized intraperitoneally and in footpads with antigen preparation in complete Freund's adjuvant. Two booster immunizations were given with antigen in incomplete Freund's adjuvant, followed by two booster immunizations with antigen alone. Boosts

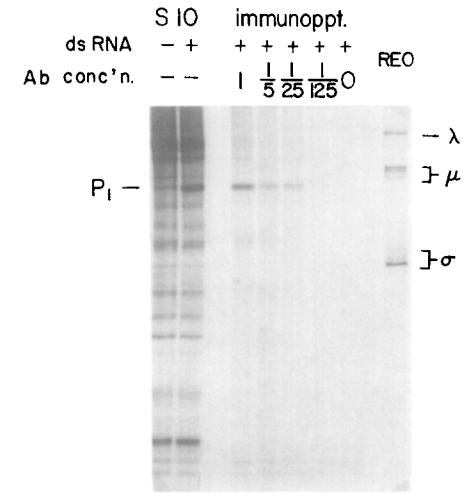
were administered at three to six week intervals. Spleens were removed from immunized mice three days after the last boost and splenocytes were fused to nonsecretor P3X63-Ag8.653 myeloma cells using polyethylene glycol (16,17). Fused cells were maintained under hypoxanthine/aminopterin/thymidine selection conditions. Hybridoma cultures were fed with normal mouse spleen cells on days 2, 6, and 10. Supernatant solutions of hybridoma culture medium were initially assayed for antibody against $P_1$ using a solid phase radioimmune assay in microtiter dishes; probable positive cultures were then further tested using the radioimmune precipitation assay described below. Positive hybridoma cultures secreting anti-$P_1$ immunoglobulins were cloned by limiting dilution and amplified. Monoclonal antibodies to $P_1$ were concentrated from hybridoma culture medium supernatant solutions by precipitation with ammonium sulfate at 50% saturation.

Radioimmune Precipitation Assays. In vitro [$\gamma$-$^{32}$P] ATP-mediated phosphorylation of protein $P_1$ present in S-10 extracts prepared from either IFN-treated human amnion U cells or mouse $L_{929}$ cells was carried out as previously described (14,18). $^{32}$P-labelled protein $P_1$ was immunoprecipitated with either polyclonal or monoclonal antibody preparations by incubation of the reaction mixture with antibody at 4°C for 14 to 18 h. Formalin fixed Staphylococcus aureus Cowan I strain was then added to the mixtures and incubation was continued on ice for an additional 45 min. Immunoprecipitates were then collected by centrifugation at 10,000 x g and washed three times with ice-cold STN buffer (150 mM NaCl; 10 mM Tris-HCl, pH 8.0; 0.5% Nonidet P-40). Washed immunoprecipitates were suspended in $NaDodSO_4$ sample extraction buffer (18), boiled for 4 min., and centrifuged; the supernatant solutions were analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis (18). Immunoprecipitation was quantitated by scanning autoradiograms at 550 nm with a Gilford spectrophotometer equipped with a linear transport accessory.

## RESULTS AND DISCUSSION

The phosphorylation of the IFN-induced phosphoprotein $P_1$ and the $\alpha$ subunit of protein synthesis initiation factor eIF-2 is believed to be catalyzed by an IFN-induced protein kinase activity which is associated with protein $P_1$ (5,12). As part of our study of the $P_1$/eIF-2$\alpha$ protein kinase, we describe in this communication the production and characterization of monoclonal and polyclonal antibodies to the IFN-induced phosphoprotein $P_1$ from human amnion U cells.

The ability of polyclonal antibodies present in serum from a rabbit immunized with human protein $P_1$ to immunoprecipitate $^{32}$P-labelled $P_1$ from an extract of human U cells is shown in Figure 1. Human protein $P_1$ was selectively immunoprecipitated in an antibody concentration-dependent manner. At saturating antibody concentrations, greater than 70% of the phosphorylated $P_1$ present in an S-10 extract from IFN-treated U cells was immunoprecipitated. Rabbit antiserum raised to protein $P_1$ from human U cells also recognized protein $P_1$ from mouse $L_{929}$ cells. The amount of protein $P_1$ immunoprecipitated at various antibody concentrations from extracts of mouse $L_{929}$ cells as compared to extracts of



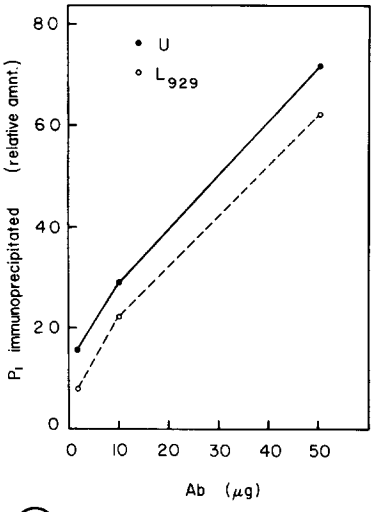




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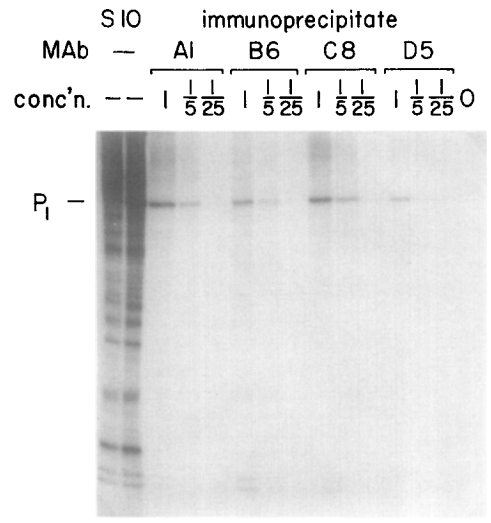
Figure 1. Immunoprecipitation of $^{32}P$-labeled protein $P_1$ from extracts of IFN-treated human amnion U cells with rabbit antiserum raised to protein $P_1$ purified from U cells. Conditions were as described under "Materials and Methods." Antibody concentrations are represented in relative amounts; a saturating concentration is designated 1.

Figure 2. Immunoprecipitation of $^{32}P$-labeled protein $P_1$ from cell-free extracts of IFN-treated human amnion U cells and from cell-free extracts of IFN-treated mouse $L_{929}$ cells with rabbit antiserum raised to human protein $P_1$. Immunoprecipitated $P_1$ was quantitated by scanning autoradiograms as described under "Materials and Methods." Relative amounts of human and of mouse protein $P_1$ immunoprecipitated were normalized to the total amount of $^{32}P$-labelled protein $P_1$ in each extract. Antibody concentrations are expressed as micrograms of immunoglobulin protein purified from immune serum by chromatography on protein A-Sepharose.

human U cells is quantitated in Figure 2. Polyclonal rabbit anti-$P_1$ serum generated against human $P_1$ immunoprecipitated mouse $P_1$ with nearly equal efficiency (Fig. 2).

Monoclonal antibodies against protein $P_1$ were produced from hybridoma cultures obtained by fusion of P3X63-Ag8.653 myeloma cells with splenocytes from BALB/c mice immunized with $P_1$ purified from human U cells. Monoclonal antibodies secreted by four representative cloned hybridoma lines were selected for detailed characterization. All four of the hybridoma lines produced antibodies of the IgM class. These IgM antibodies, like certain other IgM antibodies which have been described (19), specifically bound to S. aureus protein A. As shown in Figure 3, monoclonal antibodies A1, B6, C8, and D5 all immunoprecipitated human protein $P_1$ in a concentration-dependent manner. However, they differed

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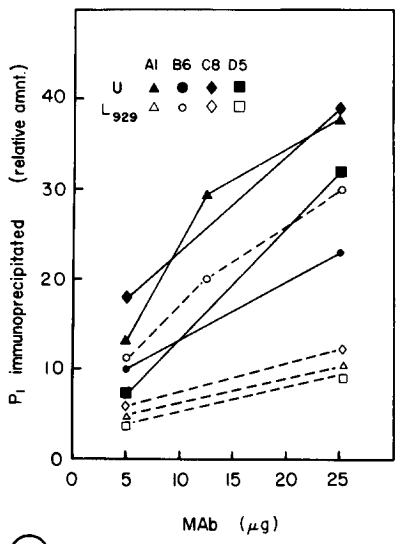


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Figure 3. Immunoprecipitation of $^{32}P$-labeled protein $P_1$ from extracts of IFN-treated human amnion U cells with various monoclonal antibodies. Monoclonal antibodies A1, B6, C8 and D5 were prepared from hybridomas produced using splenocytes of BALB/c mice immunized with protein $P_1$ purified from human U cells. Antibody concentrations are represented in relative amounts; a saturating concentration is designated 1.

Figure 4. Immunoprecipitation of $^{32}P$-labeled protein $P_1$ from cell-free extracts prepared from IFN-treated human amnion U cells and from IFN-treated mouse $L_{929}$ cells with monoclonal antibodies produced against human protein $P_1$. Quantitation of immunoprecipitated $P_1$ was as described in the legend for Figure 2. Antibody concentrations are expressed as micrograms of protein purified from hybridoma culture supernatant solutions by precipitation with ammonium sulfate.

from each other in their efficiency of immunoprecipitation of protein $P_1$. Monoclonals A1 and C8 were more efficient than B6 and D5 in the S. aureus protein A immunoprecipitation assay with protein $P_1$ from human amnion U cells.

Monoclonal antibodies A1, B6, C8, and D5 generated against human $P_1$ were also examined for their ability to recognize mouse $P_1$ (Fig. 4). Monoclonals A1 and C8, which efficiently recognized protein $P_1$ from human U cells, only poorly recognized protein $P_1$ from mouse $L_{929}$ cells. Monoclonal D5 did not recognize mouse $P_1$. By contrast, mouse $P_1$ was efficiently recognized by monoclonal B6.

Two forms of protein $P_1$ have been described which differ from each other by their phosphorylation patterns. In protein $P_1$ from mouse cells, a major phosphopeptide designated $X_{ds}$ is phosphorylated in the presence of dsRNA but not in the absence of dsRNA (20,21). As an approach to examining whether any of the monoclonal antibodies recognize subpopulations of the $P_1$ protein kinase,

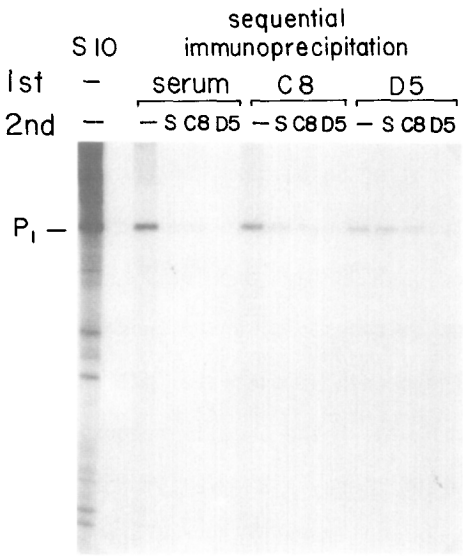


Figure 5. Sequential immunoprecipitation of $^{32}$P-labeled protein $P_1$ from cell-free extracts of IFN-treated human amnion U cells with rabbit antiserum and with monoclonal antibodies raised against human protein $P_1$. Supernatant fractions obtained after precipitation with S. aureus of the immune complex formed with the indicated first antibody (rabbit antiserum or monclonal antibody C8 or D5) were incubated with a second antibody preparation, either rabbit antiserum (S) or monoclonal C8 or D5. Antibodies were used at saturating concentration, as determined by the experiments shown in Figures 1 and 3. Immunoprecipitates from the first (-) and second (S, C8, D5) antibody incubations were analyzed by gel electrophoresis and autoradiography.

polyclonal and monoclonal antibodies were examined in a sequential manner for their ability to immunoprecipitate protein $P_1$. Aliquots of S-10 extract containing $^{32}$P-labelled protein $P_1$ were first incubated with a saturating concentration of one antibody. Immune complexes were collected by centrifugation following the addition of S. aureus; the supernatant fractions were then incubated for a second time with either the same or a different antibody. As shown in Figure 5, both the polyclonal serum antibodies and the monoclonal antibody C8 immunoprecipitated most of the phosphorylated $P_1$. After immunoprecipitation using polyclonal serum as the first antibody, no $P_1$ remained in the supernatant which could be immunoprecipitated with a second antibody addition, either serum or C8 or D5 (Fig. 5). Similar results were obtained when C8 was used as the first antibody rather than serum. By contrast, monoclonal antibody D5 at saturation immunoprecipitated only about 15 to 20 % of the amount of $P_1$ immunoprecipitated by polyclonal serum antibodies or monoclonal C8 as

determined by densitometric scanning of the gel autoradiogram (Fig. 5). This result suggests that monoclonal D5 may recognize a distinct subpopulation of $P_1$. This notion is supported by the observation that monoclonal D5 was the only antibody, either monoclonal or polyclonal, which immunoprecipitated protein $P_1$ after $P_1$ had been denatured by boiling in the presence of 0.1% $NaDodSO_4$ (data not shown). Thus, monoclonal D5 may conceivably recognize a subpopulation of native $P_1$ which includes epitopes similar to those present on denatured $P_1$.

The results reported herein demonstrate that rabbit immune serum raised against protein $P_1$ from human amnion U cells recognized both human U and mouse $L_{929}$ $P_1$ with comparable efficiency. However, monoclonal antibodies distinguished between the $P_1$ proteins from these two species. Penn and Williams (22) likewise observed that an IgM monoclonal raised to $P_1$ from human T98G cells did not recognize $P_1$ from mouse $L_{929}$ cells. Both polyclonal and monoclonal antibodies immunoprecipitated human $P_1$ and a protein kinase activity which could subsequently catalyze the phosphorylation both of $P_1$ and of added eIF-2α (data not shown). Hovanessian and coworkers (23) also observed that a monoclonal antibody raised to protein $P_1$ from human Daudi cells immunoprecipitated a protein kinase that phosphorylates protein $P_1$ and eIF-2α. These results are consistent with the notion that protein $P_1$ is the kinase which, upon activation, catalyzes the phosphorylation of itself and eIF-2α. These antibodies should prove useful in the further elucidation of the role of the $P_1$/eIF-2α kinase in the actions of interferon.

## ACKNOWLEDGMENT

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