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Interferon-Induced Human Protein MxA Is a GTPase Which Binds Transiently to Cellular Proteins

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MxA is an abundant and ubiquitous cytoplasmic protein induced by alpha/beta interferon in human cells. Upon full induction, it can constitute 0.5 to 1% of cytosolic proteins. MxA can bind elements of the cytoskeleton, such as actin and tubulins, and several larger cellular proteins. However, these protein-protein interactions seem to be transitory. The human MxA protein contains a tripartite GTP-binding domain consisting of GxxxxGKS, DxxG, and TKxD, where x is any amino acid. It is shown here that the native MxA protein has GTPase activity (GTP→GDP) when purified by immunoprecipitation with affinity-purified polyclonal antibodies directed against the C-terminal domain of MxA. The GTPase activity is greatly diminished by polyclonal antibodies directed against the N-terminal domain of MxA (the domain which contains the GTP-binding consensus elements). Amino acid substitution within the GTP-binding domain abolished the GTPase activity of the mutated MxA protein expressed in transfected CHO cells. The reaction is specific for GTP, and the approximate K_m is 0.1 mM. The reaction has an absolute requirement for Mg^{2+}. The turnover number is approximately 70 molecules of GTP hydrolyzed per min per MxA molecule. It is suggested that the human MxA protein has certain characteristics of the stress proteins.

Mx proteins belong to a family of ubiquitous proteins with antiviral potential induced in mammalian cells by alpha interferon (IFN-α) and IFN-β, by double-stranded RNA, and by some viruses (1, 3, 6, 8, 14). Mx proteins possess structural analogies with the yeast protein VPS1p/SPO15 (15, 24), the dynamin of rat brain (13), and its cognate homolog, the fly shibire protein (2, 23). The sequence conservation of the proteins suggests the presence of two separate functional domains—a more highly conserved N-terminal guanosine nucleotide (GTP)-binding domain and a more variable C-terminal domain. Biochemical evidence and mutant analysis indicate that the proteins related to Mx in sequence are involved in protein trafficking. Despite these analogies, the exact function and the mechanism of action(s) of Mx proteins are incompletely understood. Up till now, the characterization of Mx proteins relied mainly, if not exclusively, on their biological activity as potential antiviral agents (14). However, this approach does not help to analyze whether Mx proteins serve basic cellular functions.

The experimental results reported here represent a first attempt to characterize an Mx protein, the IFN-induced human MxA (huMxA) protein (a cytoplasmic protein called p78 or Mx1 in reference 7) at the biochemical level. The results support a role for GTP and protein-protein interactions in Mx function. Moreover, the results suggest that Mx proteins have several of the properties of stress proteins.

MATERIALS AND METHODS

Cells, antibodies, and IFN. Human embryonic lung (HEL) cells (catalog no. 02-031; Flow Laboratories 2002) were grown in minimal Eagle's medium-Earle salts supplemented with 10% fetal calf serum. Antiserum to huMxA protein was obtained from rabbits immunized with recombinant huMxA protein expressed in *Escherichia coli* and purified to homogeneity as described elsewhere (21). The immunoglobulins of this antiserum were further separated into two fractions by affinity chromatography with recombinant huMxA protein or huMxA C-terminal fragments immobilized on Affigel (21). The N-terminal polyclonal antibodies recognized epitopes situated between amino acids 1 and 430, and the C-terminal polyclonal antibodies recognized epitopes situated downstream of amino acid 430 of the huMxA protein. The recombinant human IFN-αB/D (rHuIFN-αB/D) was produced in yeast cells and purified by immunoaffinity chromatography to a purity of >95%. rHuIFN-αB/D comprises amino acids 1 to 60 from HuIFN-αB, 61 to 92 from HuIFN-αD, and 93 to 166 from HuIFN-αB (and not 61 to 166 from HuIFN-αD, as published previously [5]).

Transfected CHO cell lines. The complete coding region for the huMxA (or p78) protein (7) was cloned between the promoter region of the murine cytomegalovirus major early gene and a β-globin-derived polyadenylation signal. An MxA mutant was prepared by site-directed mutagenesis, replacing the lysine of the first motif of the GTP-binding site (GxxxxGKS) with an asparagine (GxxxxGNS). The expression plasmid and the constructs will be described in detail elsewhere (4). CHO cells were transfected, and the clones with the highest constitutive expression of MxA or of the mutant were selected for immunoprecipitation and the GTPase assay.

Protein binding to MxA. Cultures of HEL cells were incubated for 18 h in normal medium as control cells or in medium containing 300 IU of rHuIFN-αB/D per ml to accumulate unlabeled (and very stable) MxA. The cultures were then washed and further incubated for 4 h in the absence of IFN to minimize further MxA synthesis. Finally, the cultures were pulse labeled with 20 μCi of [^{35}S]methionine per ml for 60 min in Hanks buffer supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). Cells were collected and lysed for 10 min at 4°C in hypotonic buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NH_4Cl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. The lysate was clarified by centrifugation at 600 × *g*, and the supernatant was stored at −20°C. Thawed lysate supernatants were centrifuged at 11,000 × *g* for 5 min, and the resulting supernatant was adjusted to 1% Nonidet P-40, 1% deoxy-

cholate, and 0.1% sodium dodecyl sulfate (SDS) and kept for 5 min at 4°C. Samples were then diluted with two volumes of phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 0.5% Nonidet P-40 prior to addition of 5 μl of rabbit antiserum to huMxA or of normal rabbit serum. After 30 min of constant agitation, 50 μl of protein A-Sepharose CL-4B (Pharmacia LKB; a 50% suspension in 10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.5% Nonidet P-40) was added, and the samples were further agitated for 60 min. Immunoprecipitates were washed by two centrifugations through a sucrose cushion (1 M sucrose, 0.5 M NaCl, 0.5% Nonidet P-40), four times in phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 0.5% Nonidet P-40, and once in phosphate-buffered saline. The proteins recovered in the immunoprecipitates were analyzed on SDS-polyacrylamide gels and visualized by fluorography.

GTPase activity of huMxA in immunoprecipitates. HEL cell lysates were prepared as described above, with two modifications: the step involving the mixture of detergents was omitted, and the immunoprecipitation was carried out with affinity-purified polyclonal antibodies recognizing the C terminus of huMxA. The last washing step was done in 20 mM HEPES (pH 7.2)–5 mM $MgCl_2$–75 mM KCl. The immunoprecipitates were then incubated with 20 μl of buffer containing 20 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 75 mM KCl, 5 mM mercaptoethanol, and 40 μCi of an α-^{32}P-labeled nucleoside triphosphate (NTP). The concentration of unlabeled NTP was 0.2 mM. Aliquots taken at 0, 20, 40, and 60 min of incubation at 37°C were chromatographed on polyethyleneimine-cellulose thin-layer plates in 1 M LiCl–0.5 M HCOOH. Quantitative measurements were done by counting the radioactivity of individual spots.

Nucleotide sequence accession number. The primary nucleotide sequence encoding the huMxA protein described in this article and earlier (7) can be found in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M33882.

RESULTS

Abundancy and protein interaction. The huMxA protein was induced over 24 h in HEL cells in the presence of saturating concentrations of rHuIFN-αB/D. huMxA protein, undetectable in control cells, constituted 0.5 to 1% of cytosolic proteins in IFN-induced cells, as measured by quantitative Western immunoblot analysis with a calibrated recombinant MxA standard, described elsewhere (21). This high abundancy was suggestive of stoichiometric interaction with cellular components rather than of a purely enzymatic function of huMxA protein.

The interaction of huMxA with cellular components was tested as follows. Cultures of HEL cells were treated with rHuIFN-αB/D until the accumulation of huMxA (a very stable protein) reached a plateau (3). They were then pulse-labeled with [^{35}S]methionine. A cytoplasmic extract was prepared for immunoprecipitation with rabbit polyclonal antibodies to huMxA protein. Cell lysates were treated with 1% Triton X-100, 1% deoxycholate, and 0.1% SDS prior to immunoprecipitation. The immunoprecipitates were washed thoroughly to minimize nonspecific interactions.

A series of proteins coprecipitated with huMxA, as revealed by the appearance of discrete high-molecular-weight bands after autoradiography of SDS gels (Fig. 1A, lane 3a). Three bands corresponded in size to tubulins α and β (45 to 50 kDa) and to actin (42 kDa), and this correspondence was confirmed by comigration in two-dimensional gel electrophoresis. All of these bands were absent in gels of immunoprecipitates from control (uninduced) cell extracts and in gels of immunoprecipitates for which a normal rabbit serum was used (Fig. 1A, lanes 1a and b and 3b). These bands were largely absent in immunoprecipitates of lysates obtained after mixing unlabeled IFN-induced cells with control cells that had been labeled with [^{35}S]methionine (Fig. 1A, lanes 2a and b), indicating that these interactions had mainly occurred prior to cell lysis. However, a faint band of actin is visible in lane 2a (Fig. 1A), which may be explained by the stickiness of actin or by a true interaction occurring after lysis of the mixed cells.

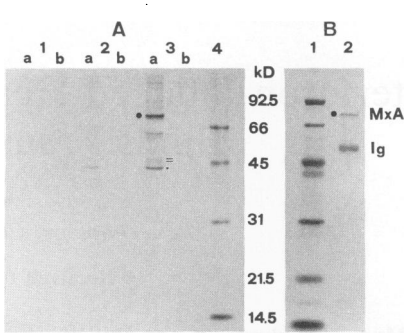

FIG. 1. Electrophoretic analysis of protein binding to huMxA. (A) Fluorograph of proteins in immunoprecipitates from (lanes 1) ^{35}S-labeled control cells, (lanes 2) ^{35}S-labeled control cells mixed with unlabeled IFN-induced cells prior to lysis, and (lanes 3) ^{35}S-labeled IFN-induced cells. Lane 4, ^{14}C-labeled size markers (in kilodaltons). Immunoprecipitation was done with (lanes a) rabbit antiserum to huMxA or (lanes b) normal rabbit serum. The positions of actin and tubulins are noted with a small dot and dashes, respectively. The large dot marks the MxA protein. (B) Electrophoretic analysis and Coomassie blue staining of proteins from immunoprecipitates used for the GTPase assay. Lane 1, size markers; lane 2, immunoprecipitate from HEL cells induced by IFN. The positions of huMxA protein and of the large chain of immunoglobulins (Ig) used for immunoprecipitation are indicated.

Taken together, these results suggested true interaction between huMxA and certain cytoskeletal components and possibly other proteins. The coprecipitated proteins, however, represented only a minor component of the Mx immunocomplex, since they could not be visualized by Coomassie blue staining (Fig. 1B). By fluorescence microscopy, huMxA is detectable in IFN-induced cells as a uniform punctate stain throughout the cytoplasm (19), suggesting an association with vesiclelike structures and suggesting also that the interactions with elements of the cytoskeleton were only transitory.

Function of the GTP domain. In preliminary studies designed to test the hypothesis that Mx is a GTP-binding protein, lysates of induced cells were separated on denaturing polyacrylamide gels. They were then electrophoretically transferred to a nitrocellulose sheet, renatured, and reacted with α-^{32}P-labeled GTP. The results were unimpressive (not shown). Moreover, a procedure which allows the detection of [^{32}P]GTP bound to Ras protein (protein containing the GTP-binding consensus elements) after in vivo labeling (16) gave negative results when applied to huMxA. Because of the negative outcome of those experiments, attempts to detect and characterize a GTPase activity associated with MxA were initiated.

Intrinsic GTPase activity of MxA protein. MxA has been expressed in *E. coli*, but the recombinant protein is accumu-

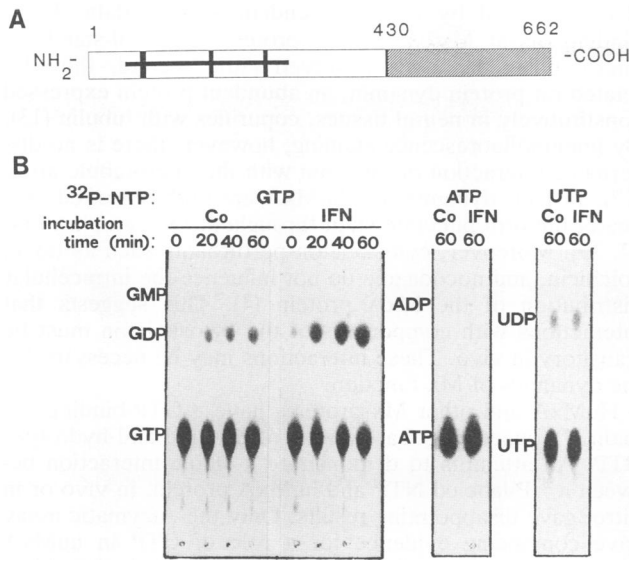

FIG. 2. (A) Map of the huMxA protein, showing the position of the three consensus elements of the GTP domain (vertical lines). Immunoprecipitates for GTPase activity were prepared by using polyclonal antibodies directed against the hatched portion of the protein. The horizontal bar covers the region of highest homology between Mx proteins and related proteins. (B) GTPase activity of huMxA immunoprecipitates. The NTPase assay was performed as described in Materials and Methods with immunoprecipitates from control cell extracts (Co) lacking huMxA and from IFN-induced cell extracts (IFN) containing huMxA. The concentration of the NTP used was 0.2 mM. Aliquots taken at the indicated times were chromatographed on polyethyleneimine-cellulose thin-layer plates. Autoradiographs of thin-layer plates are shown.

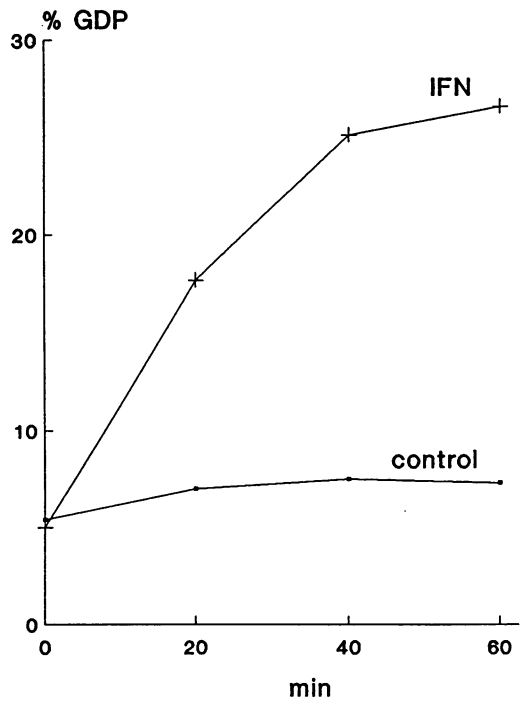

FIG. 3. Time course of GTP→GDP hydrolysis in a representative experiment. Immunoprecipitates from IFN-induced HEL cells or from an equivalent amount of control HEL cells were subjected to the GTPase assay. The radioactive spots corresponding to GTP and GDP were localized by autoradiography, and the radioactivity was determined by scintillation counting.

lated in inclusion bodies as aggregates of misfolded protein unsuitable for purification and enzymatic studies (21). Because of the difficulty of purifying MxA in its native state, it was decided to immunoprecipitate Mx from IFN-induced HEL cells and to examine its activity while it was still bound to polyclonal antibodies on protein A-Sepharose beads. The affinity-purified polyclonal antibodies used for immunoprecipitation recognized epitopes situated downstream of amino acid 430, that is, outside of the putative enzymatic site (Fig. 2A).

Immunoprecipitates were assayed for NTPase activity as described in Materials and Methods. Figure 2B shows that huMxA protein had substantial GTPase activity and no measurable ATPase or UTPase activity. The [α-^{32}P]GTP substrate was selectively hydrolyzed to GDP. No further hydrolysis to GMP or to guanosine and inorganic phosphate was seen. Figure 3 shows the kinetics of the reaction, which reached a plateau after approximately 40 min of incubation at 37°C.

Factors influencing the GTPase assay are summarized in Table 1. The GTPase activity was completely dependent on Mg^{2+} ions. Addition of Ca^{2+} ions did not influence the reaction. Addition of pure tubulin (0.1 mg/ml), which may interact with huMxA, did not enhance the GTPase activity of huMxA. Similar background values were obtained when the polyclonal antibodies were omitted from the immunoprecipitation procedure and when extracts from noninduced cells (no huMxA in the extract) were used, showing that the huMxA protein was essential for the activity. The GTPase activity was greatly reduced when MxA was immunoprecipitated with an affinity-purified polyclonal antibody directed against the N terminus of MxA, a region comprising the GTP-binding domain. All immunoprecipitates contained comparable amounts of MxA, as determined by quantitative Western blot analysis. These results suggested that huMxA had intrinsic GTPase activity and that the N terminus of huMxA was essential for this catalytic activity.

A mutation was introduced in MxA cDNA, replacing the basic lysine of the first sequence motif (GxxxxGKS) with an uncharged polar asparagine residue (MxA-Asn-83). MxA and MxA-Asn-83 cDNAs were transfected into CHO cells.

TABLE 1. Relative effects of assay conditions on GTPase activity

Cell extract	Antibody	GTPase assay mix	Relative GTPase activity (% of control)
IFN-induced HEL cells (control)	C terminus	Complete cocktail	100
	None	Complete cocktail	<5
	N terminus	Complete cocktail	<5
	C terminus	Mg^{2+} omitted	<5
	C terminus	Ca^{2+} added	80
	C terminus	Tubulin[a] added	65
CHO-huMxA (control)	C terminus	Complete cocktail	100
CHO-huMxA-Asn-83	C terminus	Complete cocktail	<5

[a] Tubulin from rat brain was purified as described before (22), including the final DEAE-Sephadex step, and 2 μg was added to the assay mix.

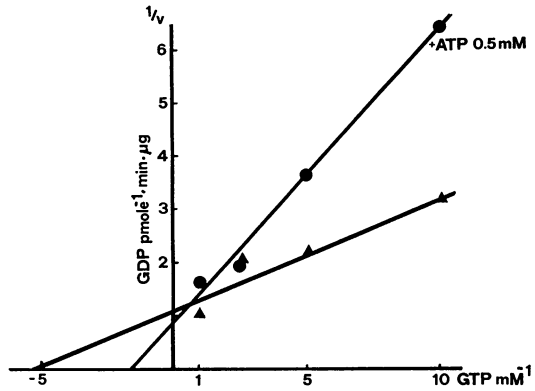

FIG. 4. Competitive inhibition of the GTPase activity of huMxA by ATP. The concentration of ATP was 0.5 mM. The double-reciprocal plots of the GTP concentration versus the amount of GDP formed per minute per microgram of huMxA are represented (taken as an average of two independent experiments).

The CHO clones with the highest constitutive expression of the cDNA product were selected for immunoprecipitation. No GTPase activity was measurable in immunoprecipitates of the mutated MxA-Asn-83 protein (Table 1). These results strengthened the conclusion that the GTPase activity was an intrinsic property of MxA.

Enzymatic parameters. Competition experiments with 0.4 mM GTP and 2 mM NTP indicated that NTPs partially inhibited the GTPase activity in the following decreasing order of potency: ATP (50 to 70% inhibition) ≥ ITP > UTP ≥ XTP > CTP. ATP, which was not hydrolyzed by huMxA, was shown to act as a competitive inhibitor (Fig. 4). The K_m for GTP was calculated from double-reciprocal plots (Fig. 3C) of the GTP concentration versus the amount of GDP formed per minute. The K_m of the MxA-GTPase reaction was estimated to be 0.1 mM, an average value obtained from several independent experiments. The amount of MxA in each GTPase assay mix was determined at the end of the reaction by quantitative immunoblot analysis of proteins in the immunoprecipitates (21). This determination and the calculation of the maximal velocity (V_{max}) from double-reciprocal plots allowed the calculation of the approximate turnover rate, which was 70 molecules of GTP hydrolyzed per min per molecule of huMxA.

DISCUSSION

Cloning of cDNA has given insights into the mechanism of action of Mx proteins and their relationship to other proteins. Mx proteins belong to a superfamily with representatives in *Saccharomyces cerevisiae* (15, 24), *Drosophila melanogaster* (2, 23), and the rat (13). Genetic and biochemical evidence shows that these Mx sequence-related proteins participate in intracellular transport and sorting mechanisms in relation with proteins. These considerations suggest that Mx proteins might have important functions in protein trafficking. The results discussed below are in line with this hypothesis.

The observation that huMxA protein constitutes up to 1% of cytosolic proteins in fully induced diploid fibroblasts suggested stoichiometric interactions with cellular components. The studies on protein-protein interactions indicated that huMxA is capable of binding to elements of the cytoskeleton and possibly to other proteins. This conclusion is also supported by two independent sets of data. First, binding of rat Mx2 and Mx3 proteins to taxol-stabilized microtubules has been observed (10). Second, the Mx-related rat protein dynamin, an abundant protein expressed constitutively in neural tissues, copurifies with tubulin (13). By immunofluorescence staining, however, there is no discernable interaction of dynamin with the microtubule array (17). In fact, dynamin and huMxA are both observed as a nearly uniform punctate stain throughout the cytoplasm (4, 17, 19). Moreover, cytoskeleton perturbants such as taxol, colchicin, and nocodazole do not influence the intracellular distribution of the MxA protein (4). This suggests that interactions with components of the cytoskeleton must be transitory in vivo. These interactions may be necessary for the dynamics of Mx function.

HuMxA and other Mx proteins have a GTP-binding domain (7), suggesting that huMxA might bind and hydrolyze GTP. All attempts to demonstrate a stable interaction between a ^{32}P-labeled NTP and huMxA protein, in vivo or in vitro, gave disappointing results. Only the enzymatic assay gave convincing evidence for a role of GTP in huMxA function. The assay involves the purification of huMxA protein in its native state by immunoprecipitation. The GTPase activity of huMxA has been measured while it was still bound to polyclonal antibodies on protein A-Sepharose beads, and it represents an intrinsic activity of huMxA protein for the following reasons. The GTPase activity depended on the use of affinity-purified polyclonal antibodies directed against the C-terminal domain of huMxA, whereas polyclonal antibodies directed against the N-terminal domain (the GTP-binding domain) completely abolished the GTPase activity. These polyclonal antibodies were obtained from rabbits immunized with the recombinant huMxA protein (21), eliminating the possibility that they were also directed against a human protein coprecipitating with the huMxA protein. Finally, an amino acid substitution within the GTP-binding motif destroyed the GTPase activity. These studies have also established that GTP is the physiological substrate. The K_m for GTP is approximately 0.1 mM, well below the intracellular concentration of GTP, which is in general 1 mM or higher (20). However, the GTP-binding domain can accommodate other NTPs, such as ATP, which acts as a competitive inhibitor. Since the intracellular concentration of ATP is higher than that of GTP (20), ATP might play a regulatory role on huMxA function, provided that huMxA and these high concentrations of ATP are localized in the same cellular compartment.

Recently, Nakayama et al. (12) succeeded in purifying an active recombinant mouse Mx1 expressed in *E. coli*, confirming independently the GTPase activity of Mx proteins. A mutation in the GTP-binding site of Mx1 also greatly diminished the GTPase activity. However, the turnover rate is 5.8 molecules of GTP hydrolyzed per min per molecule of mouse Mx1, compared with 70 molecules for the human MxA, and substantial amounts of ATP were hydrolyzed by the recombinant mouse Mx1. The turnover rate of 70 molecules of GTP hydrolyzed per min per molecule of huMxA appears to be low but is comparable to the value of 20 to 40 molecules of GTP hydrolyzed per min per molecule of dynamin that can be calculated for the GTPase activity of the Mx-related rat protein dynamin (13, 18). The turnover value of 20 to 40 molecules is only observed when dynamin is activated by microtubules. This comparison and the close resemblance in turnover number suggests two hypotheses: (i) the C-terminal polyclonal antibodies have stimulated the intrinsic GTPase activity of huMxA in the assay by mimick-

ing an interaction with a putative cellular protein; or (ii) the C-terminal sequence of huMxA plays a role in its function. It has already been shown that mutations in the C-terminal sequence of Mx proteins can affect their antiviral activity (14).

The huMxA protein and possibly all Mx homologs have several properties in common with stress proteins (11): ubiquity, abundance, induction by stress conditions (e.g., activation of the cytokine network, double-stranded RNA, some viral infections), binding to proteins, sequence conservation, and low NTPase activity. By comparison, the intrinsic ATPase activity of heat shock proteins is very low, approximately 0.1 to 1 molecule of ATP hydrolyzed per min per monomer. However, this rate can be stimulated by up to 50-fold by protein-protein interactions (9), which nearly approaches the rate of huMxA protein.

In conclusion, Mx proteins may be important to maintain adequate functioning of the cell by protein-protein interactions during cellular stress, the GTP-GDP cycle being mediated by its intrinsic GTPase activity. Perhaps Mx proteins interact with specific "foreign" proteins, of viral origin, to exert their antiviral action (14).

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