The P55 Protein Affected by *v-mos* Expression Is Vimentin

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Rabbit antiserum prepared against a cyclic 19-amino-acid peptide predicted from the sequence of the viral *mos* gene (*v-mos*) of Moloney murine sarcoma virus not only recognized *v-mos* gene products but also specifically detected a 55,000-*M_r* polypeptide expressed in a variety of cells that grow on solid surfaces. This normal cellular protein, previously shown to be reduced in amount in cells expressing the *v-mos* gene, was found to be the intermediate filament structural protein, vimentin. This conclusion was reached by comparing relative mobilities in denaturing gels, isoelectric points, immunoreactivities, location in the cell, and peptide maps.

Antibodies raised against synthetic peptides facilitate the isolation, purification, and characterization of proteins. The viral *mos* gene (*v-mos*) product encoded by Moloney murine sarcoma virus (Mo-MuSV) is made in trace amounts in cells transformed by typical strains of Mo-MuSV (21). Prior to the expression of large amounts of *v-mos* in recombinant DNA systems, the *v-mos* protein was not easily obtained in amounts needed for antibody production. Thus, to identify and study the *v-mos* protein, two laboratories made synthetic *mos* peptides that produce antibodies capable of detecting intracellular *v-mos* proteins (6, 21). Two peptides from the C terminus and one peptide near the N terminus of the *v-mos* protein stimulated the production of useful antibodies (6, 21).

The antisera to the N-terminal *mos* peptide (residues 37 to 55) were prepared from a peptide that was 19 amino acids long and that was cyclized by means of a disulfide bond resulting from the coupling of cysteines present at residues 39 and 55 of the *mos* peptide. Rabbit antisera to this peptide [anti-*mos*(37-55)] were shown to react with *gag-mos* and *env-mos* proteins; the specificity of the recognition was proven, since pretreatment of the antisera with excess peptide blocked the antibody recognition of *v-mos* proteins (6). Such immune complexes have proven to be especially useful for the detection of the serine/threonine protein kinase activity which appears to be an intrinsic function of *v-mos* proteins (11-15, 23, 25).

A second protein was specifically recognized by the rabbit anti-*mos*(37-55) serum (6). This protein (apparent *M_r*, 55,000; termed P55) was detected in a variety of cells grown in cultures, including cell lines from rats, mice, mink, and humans. Of interest was the finding that P55 was decreased in concentration when cells were acutely infected with Mo-MuSV strain 124 (24). The effect of the virus on P55 required a functional *v-mos* gene product, since the reduction in the steady-state level of P55 occurred only at the temperature permissive for *v-mos* effects and not at the restrictive temperature in cells infected with the temperature-sensitive *mos* mutant termed *ts110* Mo-MuSV (24; for a review on the *ts110* system, see reference 1). More recent studies showed that a marked decrease in the level of P55

correlated with the increased expression of Mo-MuSV RNA in infected cells (4). It was of interest, therefore, to determine the identity of P55.

In this report, we show that P55 is identical to the intermediate filament (IF) protein, vimentin, in its physical, biological, biochemical, and immunological properties.

The location of P55 within rat L₆E₉ myoblast cells (16) was investigated. Lysis of cells with Nonidet P-40 (NP-40) detergent-containing buffers and low-speed centrifugation showed that P55 was located primarily in the nuclear-cytoskeletal fraction, as measured by immunoblotting with anti-*mos*(37-55) serum (Fig. 1 A and B). P55 could also be detected by affinity-purified anti-*mos*(37-55) serum previously purified over a peptide affinity column; as expected, the recognition of P55 by the affinity-purified antibody was blocked by the addition of excess peptide (results not shown). The *v-mos* peptide (residues 37 to 55) was cyclized from 95% pure linear peptide, and the cyclized peptide was repurified (6). It was found to be greater than 95% pure, on the basis of high-pressure liquid chromatography analysis and amino acid composition. P55 could be detected in the soluble cytoplasmic fraction but in very low amounts, as measured by 30 min of pulse-labeling of L₆E₉ cells with [³H]leucine (6). Sonication of cells labeled in this manner released significantly more P55 into the detergent-soluble fraction, indicating that P55 was of a particulate nature (see below).

Given the relatively insoluble nature of P55, we reasoned that it may be a component of the cytoskeleton. A series of antibodies which recognize actin, tubulin, and vimentin were tested. P55 and vimentin had the same relative mobilities in sodium dodecyl sulfate (SDS) gels. Both polyclonal and monoclonal antibodies (Fig. 1C) prepared against vimentin recognized P55 preparations. The polyclonal serum was made against vimentin purified from BHK-21 cells (9). The monoclonal antibody against vimentin was purchased from a commercial source (Lab Systems, Morton Grove, Ill.).

If in fact vimentin and P55 are the same protein, then the removal of vimentin with anti-vimentin antibody should also remove P55 detectable with anti-*mos*(37-55) sera. In this experiment, L₆E₉ cells were pulse-labeled for 30 min with [³H]leucine, sonicated, and then extracted with NP-40-containing buffer. The detergent-soluble fraction was treated with normal rabbit serum, and the antibody was harvested

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FIG. 1. Subcellular fractionation of P55 and its comigration with vimentin. About 80 to 90% confluent monolayer cells of rat L₆E₉ myoblasts were lysed by Dounce homogenization in 30 mM Tris hydrochloride (pH 7.5)–125 mM KCl–5.7 mM MgCl₂–3.6 mM CaCl₂–0.5 mM disodium EDTA–0.5% NP-40–0.1% sodium deoxycholate (17). The extract was centrifuged at 5,000 rpm in a Sorvall RC-3 centrifuge (HG-4L rotor) for 10 min. The pellet was suspended in 1× sample buffer (6). A portion of the 5,000-rpm supernatant was made 1× with respect to sample buffer by the addition of appropriate volumes of 5X sample buffer. Samples from equal cell volumes were separated on an 8% SDS–polyacrylamide gel and immunoblotted as described earlier (2, 27). (A) Anti-*v-mos* peptide serum. (B) Anti-*v-mos* serum blocked with the *v-mos* peptide. Lanes: 1, 5,000-rpm pellet; 2, 5,000-rpm supernatant. (C) Recognition of P55 in an immunoblot of a whole-cell extract by polyclonal (lane 1) or monoclonal (lane 2) anti-vimentin antibodies. The monoclonal antibody was an immunoglobulin G1 subtype. Therefore, goat anti-mouse immunoglobulin G1 was used before reaction with ¹²⁵I-protein A. Polyclonal antibodies were prepared against vimentin purified from BHK-21 cells (9). V, Vimentin; M, *M_r* (in thousands).

by precipitation with Pansorbin. The supernatant fluid was divided into two portions: one portion was immunoprecipitated with anti-vimentin serum, and the other was immunoprecipitated with anti-*mos*(37-55) serum. Anti-vimentin and anti-*mos*(37-55) sera immunoprecipitated the same-size polypeptide (Fig. 2, lanes 1 and 4, respectively). Anti-tubulin and anti-actin recognized different-size proteins (results not shown). When clearing was done with anti-vimentin rabbit serum instead of normal rabbit serum, P55 recognized by anti-*mos*(37-55) serum was completely removed (Fig. 2, lane 2), leading to the conclusion that P55 is in fact vimentin.

To confirm the cytoplasmic location of P55, we examined NIH 3T3 cells by indirect immunofluorescence with our anti-*mos*(37-55) serum. The location of P55 was found to be cytoplasmic, with higher concentrations around the nucleus of the cell (Fig. 3). The pattern of staining was consistent with that typically found with anti-vimentin antibodies (Fig. 3A and C). Colchicine treatment of spread fibroblasts results in the retraction of the majority of IFs into a juxtanuclear position as microtubule depolymerization takes place (9).

Therefore, 3T3 cells were subcultured on glass cover slips and kept in the incubator overnight to attain a fully spread configuration. These cells were then treated with 5 μg of colchicine per ml of culture medium for 3.5 h at 37°C. Subsequently, they were examined by indirect immunofluorescence with anti-*mos*(37-55) antibody. Once again this antibody (Fig. 3B) exhibited the pattern expected for an antibody raised against vimentin (Fig. 3D). As a further control, the fluorescent vimentin pattern was abolished upon blocking of the anti-peptide antibody with an excess of the *v-mos* peptide (results not shown).

IF preparations were also examined by one-dimensional gel electrophoresis and immunoblotting to determine which molecular species anti-*mos*(37-55) serum detects. IF-enriched cytoskeletal preparations were made from cultured BHK-21 and NIH 3T3 cells (26, 32). Anti-*mos*(37-55) antibody detected the same major 55-kilodalton band as did anti-vimentin antibody (data not shown).

Two-dimensional gel separation methods were also used to determine the P55 pattern (Fig. 4). The procedure of O'Farrell was used (20). Uninfected L₆E₉ cells were labeled with [³H]leucine (0.05 mCi/ml) for 3 days. A cell lysate was prepared by sonicating the cells in NP-40-containing buffer; P55 was immunoprecipitated with anti-*mos*(37-55) serum (Fig. 4A). To determine the location of P55 among the total cellular proteins in a two-dimensional gel pattern (Fig. 4B), we did a mixing experiment. In this case, total cellular proteins previously labeled with [³H]leucine were mixed with the anti-*mos*(37-55) immunoprecipitate (Fig. 4C); the addition of the latter should have enhanced the spot of P55

FIG. 2. Immunoprecipitation of P55 with anti-*mos*(37-55) and anti-vimentin sera. [³H]leucine-labeled, sonicated L₆E₉ cell extracts were immunoprecipitated with anti-vimentin (lane 1) or anti-*mos*(37-55) (lane 4) sera as described earlier (6). The supernatants obtained after 5,000-rpm centrifugation of Pansorbin-antibody-antigen complexes were subjected to immunoprecipitation with the second antibody. The supernatant fluid from lane 1 was immunoprecipitated with unblocked (lane 2) and blocked (lane 3) anti-*mos*(37-55) sera. V, Vimentin.

FIG. 3. Indirect immunofluorescence of NIH 3T3 cells stained with anti-*mos*(37-55) and anti-vimentin sera. (A) Anti-*mos*(37-55) antibody on normal spread 3T3 cells showing a fibrous pattern typical of vimentin-containing IFs. (B) Anti-*mos*(37-55) antibody on 3T3 cells treated with colchicine (5 μ g/ml of culture medium) for 3.5 h. Note the typical juxtannuclear IF cap which forms as the cytoplasmic microtubules disassemble (26). Arrowheads indicate the location of the cell periphery. Cells in panels A and B were stained with a 1:2 dilution of antibody and fluorescein-labeled second antibody. Cells were fixed in cold methanol and processed for indirect immunofluorescence as described previously (10, 31). Panels C and D were the same as panels A and B but with anti-vimentin antibody.

within the total pattern. A comparison of Fig. 4A and B with Fig. 4C clearly identified P55 in the two-dimensional gel pattern. This P55 spot had the isoelectric point and molecular weight of authentic vimentin, as determined by our own two-dimensional gel analysis of vimentin detected by immunoblotting with anti-vimentin antibody (results not shown) and based upon the published work of others (18).

The identity of P55 as vimentin was tested further by comparing the one-dimensional peptide maps of the two proteins isolated from cells metabolically labeled with [3 H]leucine. Bands excised from SDS gels of immunoprecipitated P55 and vimentin obtained from labeled cell extracts were digested with V8 protease by the method of Cleveland et al. (3) (Fig. 5). Vimentin and P55 produced identical patterns of fragments resulting from V8 protease treatment.

In previous published (6) and unpublished experiments, we noted that P55 was present in a variety of fibroblast cell lines from several species but absent from cells of lymphoid origin. We therefore compared the content of P55 and vimentin in several types of cells by immunoblotting. P55 and vimentin were detected in rat embryo fibroblasts and primary muscle cells but not in rat lymphocytes. In addition, P55 was absent from various myeloma cells (data not shown); these cells also lacked vimentin. These results are in agreement with the observations of Traub et al. (29), who showed that vimentin was absent in mouse MPC-11 myeloma cells and mouse MOPC-31C plasmacytoma cells. Similarly, human HL60 promyelocytic leukemia cells, shown to lack vimentin (2), also lacked P55 (data not

shown). However, HeLa cells were found to be positive for P55 and vimentin.

We have established that when cross-linked to a carrier protein, a cyclic 19-amino-acid peptide whose sequence was derived from the N-terminal region (residues 37 to 55) of the Mo-MuSV-encoded *mos* oncogene (30) stimulates the production of rabbit antibodies that recognize two entirely different proteins. The antiserum recognizes *v-mos* gene products, both *env-mos* and *gag-mos* proteins, either in immunoprecipitation or immunoblotting experiments (6). The antiserum also recognizes a very different protein which we have termed P55 (6, 24). P55 does not, however, have extensive homology with the *v-mos* protein because it is not recognized by anti-peptide antibodies raised against the other *v-mos* peptides (e.g., residues 260 to 271, 344 to 359, and 363 to 374 [6; unpublished data]). The need to identify P55 as a known cellular protein stemmed from our earlier studies which showed that P55 is one of two polypeptides that are detected in two-dimensional gels and that are reduced in concentration in cells infected with Mo-MuSV (24). A functional *v-mos* gene product was found to be necessary for the observed reduction in P55 concentration. The physiological importance of this reduction by the *v-mos* protein is not known.

In this report, we have obtained convincing evidence that P55 is vimentin. The evidence is based on several types of results, including the following: the relative M_r s of the two proteins in SDS gels (Fig. 1 and 2), their isoelectric points in two-dimensional gels (Fig. 4), their co-localization in cells

FIG. 4. Identification of P55 in cell extracts by a two-dimensional gel procedure. L₆E₉ cells were metabolically labeled with [³H]leucine (0.05 mCi/ml; 50 Ci/mmol) for 3 days. (A) P55 was immunoprecipitated with anti-*mos*(37-55) serum from sonicated NP-40 lysates and released from Pansorbin by boiling in SDS sample buffer. (B) Total cell extract was prepared by boiling the cells for 5 min in SDS sample buffer. The amount of labeled protein applied was adjusted to match the intensity of the P55 spot present in panel A; a longer exposure of panel B revealed many more protein spots. (C) Mixture of samples from panels A and B. Two-dimensional gels (20) were visualized by fluorography. The arrows identify the P55 spots. Numbers at right indicate M_r (in thousands).

determined by immunofluorescence (Fig. 3), their immunological (Fig. 2) and biochemical (Fig. 4 and 5) properties, and their distribution among several types of cells from various species. The finding of P55 in a wide range of species and cell types is also consistent with its identity as vimentin, whose structure is conserved among a large number of species (28). In addition to using anti-*mos*(37-55) serum and a polyclonal antiserum raised against purified vimentin, monoclonal anti-vimentin antibodies were also used in some of these experiments.

What, then, is the basis for the anti-*v-mos* peptide antibody recognition of vimentin? The answer may lie in the sequences of the two proteins. Table 1 compares the sequences of the mouse *v-mos* protein and hamster, pig, and human vimentin. Three short sequences of the *v-mos* protein (p37^{*mos*}), one near the N terminus and two near the C terminus, share homology with sequences of vimentin. The N-terminal *v-mos* sequence (residues 48 to 54) falls within the *v-mos* sequence at residues 37 to 55 [used to prepare our anti-*mos*(37-55) serum]. Thus, the *v-mos* sequence homology at residues 48 to 54 is found in either one or two short

FIG. 5. Partial V8 protease digestion of P55 and vimentin from metabolically labeled rat L₆E₉ myoblasts. [³H]leucine-labeled P55 and vimentin were immunoprecipitated with anti-*mos*(37-55) and anti-vimentin sera, respectively, and separated on an 8% SDS-polyacrylamide gel. The appropriate bands were excised, rehydrated, and subjected to partial digestion with 0.05 µg of V8 protease as described by Cleveland et al. (3). The protein fragments were separated on an SDS-polyacrylamide gel (12% polyacrylamide). Lanes: 1, P55; 2, vimentin. M, M_r (in thousands).

sequences near the N terminus of vimentin from all species examined thus far (Table 1). We propose that this sequence homology, together with the restricted conformation imposed by the cyclic form of the *v-mos* peptide, may explain the recognition by the anti-*v-mos* peptide serum of both the *v-mos* protein and vimentin. It is important to note that the N terminus of vimentin is not part of the conserved coiled-coil central rod structure which is present in all other IF

TABLE 1. Sequence homology of p37^{*mos*} and vimentin^a

Protein	Source	Residues	Sequence homology
* *			
<i>v-mos</i>	Mouse (Mo-MuSV)	48-54	P S V D S R S
Vimentin	Hamster	44-49	P S—T S R S
Vimentin	Pig	23-28	P S—S S R S
Vimentin	Pig	45-50	P S—T S R S
Vimentin	Human	24-29	P S—S S R S
Vimentin	Human	46-51	P S—T S R S
* *			
<i>v-mos</i>	Mouse (Mo-MuSV)	340-346	S L T G K A L
Vimentin	Pig	38-43	S L—G S A L
Vimentin	Human	39-44	S L—G D A L
* *			
<i>v-mos</i>	Mouse (Mo-MuSV)	359-365	L Q R P S A E
Vimentin	Hamster	192-198	L Q R E E A E
Vimentin	Human	194-200	L Q R E E A E

^a The *v-mos* sequence was taken from Van Beveren et al. (30). The hamster vimentin gene was sequenced by Quax et al. (22), and the human vimentin gene was sequenced by Ferrari et al. (5). Sequences of 464 and 466 amino acids, respectively, are predicted from the DNA sequences. A total of 275 N-terminal residues of pig vimentin were sequenced by Geisler et al. (7,8). —, No amino acid; *, nonhomologous amino acid residue. The standard single-letter amino acid code is used.

structural proteins, such as keratin, desmin, and neurofilament proteins (28).

The meaning of the *v-mos* protein and vimentin limited homologies is, of course, unknown. It is tempting to speculate that proteins bearing such sequence homologies may actually bind to IFs through their ionic association with vimentin. On the other hand, it is possible that the cross-reactivity of vimentin with the anti-*mos*(37-55) antibody is fortuitous and not due to the limited sequence homology discussed above. Such cross-reactions with major cytoskeleton proteins are a common problem with anti-peptide antibodies (see, for example, reference 19). We are synthesizing the relevant vimentin peptides to determine if they block vimentin recognition by anti-*v-mos* peptide antibodies.

We are currently investigating what direct effects *v-mos* gene expression has on vimentin in stably transformed cells grown in cultures. Since *v-mos* proteins have a closely associated serine/threonine protein kinase activity, it will be of interest to determine whether vimentin is a target for the *v-mos* kinase (13, 23, 25) in morphologically transformed cells.

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