Occurrence of the Tumor-specific, Calcium-binding Protein, Oncomodulin, in Virally Transformed Normal Rat Kidney Cells

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ABSTRACT

Oncomodulin, an apparently tumor-specific calcium-binding protein, has been detected in many chemically induced rat hepatomas. It is now possible to detect, by radioimmunoassay and immunofluorescence, the presence of oncomodulin in normal rat kidney cells virally transformed by avian sarcoma virus. By contrast, it was not detected in uninfected, nonneoplastic normal rat kidney cells. The protein was isolated and purified by a novel high-performance liquid chromatography procedure and shown to be identical to that isolated previously from rat hepatoma. The cellular levels of oncomodulin approached the levels of calmodulin in avian sarcoma virus-transformed normal rat kidney cells, suggesting that the total calcium-binding activity of the cell may play a role in expression of the transformed phenotype.

INTRODUCTION

A unique calcium-binding protein found in the extracts of many chemically induced rat hepatomas, but not normal liver, has been purified to homogeneity and shown to be an acidic protein with a molecular weight of 11,500, possessing 2 calcium-binding sites per molecule (9-11, 13, 15). The cellular function of this calcium-binding protein remains unknown, but it is capable of modulating the activity of calmodulin-dependent phosphodiesterase in vitro (12) and of mimicking the effects of calmodulin by releasing T51B rat liver cells from a G0-S transition block induced by low-calcium medium (1). The development of antisera to this protein purified from rat hepatoma has enabled immunological methods to establish the presence of immunoreactive material in tumors arising from a variety of chemical carcinogen-treated tissues and animal species (13, 15). Such material has not been detected in normal tissue or in nonneoplastic rodent and human cell lines by specific radioimmunoassay (13, 15). Because of its similarity to calmodulin and its apparent tumor specificity, this calcium-binding protein has been named oncomodulin (1, 15).

We now report the detection, by immunofluorescence and radioimmunoassay, of high levels of oncomodulin in NRK2 cells transformed by ASV. This protein has been isolated, purified by a novel and rapid method using HPLC, and shown to be identical to that isolated previously from the chemically induced rat hepatoma, 5123tc.

MATERIALS AND METHODS

Cell Line. Nonneoplastic NRK fibroblast-like cells were descendents

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3 The abbreviations used are: NRK, normal rat kidney; ASV, avian sarcoma virus; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline.

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PBS for 20 min and incubated 1 hr with fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Cappel Laboratories, West Chester, Pa.) diluted 1:100 in PBS. Cells were washed in PBS, mounted in antifade mounting medium (6), and examined on a Leitz Orthoplan microscope equipped with a Pleompak 2 fluorescence incidence illuminator and a standard fluorescein filter system. Both preimmune rabbit serum (100 µg IgG per ml) and oncomodulin-adsorbed serum served as immunological controls. Adsorbed serum was produced by the addition of purified rat hepatoma oncomodulin to Affigel Blue-purified rabbit anti-oncomodulin IgG (340 µl, 7.5 mg/ml) to a final concentration of 2 x 10^{-6} M and incubation for 1 hr at room temperature and then overnight at 4°C. Following dilution to 100 ng IgG per ml, the immunoprecipitate was pelleted by centrifugation at 35,000 x g for 1 hr, and the supernatant was used in the indirect immunofluorescent technique described above.

**Purification of ASV-NRK Oncomodulin.** Twenty-five g, wet weight, of ASV-NRK solid tumors carried as xenografts in athymic mice were extracted as described (9, 11). The 154 ml of supernatant were treated with ammonium sulfate to 65% saturation, and the proteins remaining in solution were precipitated by adjusting the pH to 4.0 with sulfuric acid (10). This precipitate was dissolved in 20 mM Tris-acetate, pH 8.0, and dialyzed against this same buffer. The immunoreactive material, as assessed by radioimmunoassay, in the dialyzed sample (12.5 ml) was purified by HPLC methods using 2 Beckman 10OA pumps controlled by a Model 420 programmer and monitored at 280 nm with a Beckman Model 165 UV detector. Ion exchange HPLC was performed first on an AX300 column (4.1 x 300 mm; Synchrom, Inc., Linden, Ind.) equilibrated in 20 mM Tris-acetate, pH 8.0, and eluted with a gradient (0 to 500 mM) of sodium acetate. The relevant fractions (Chart 1A) were pooled and lyophilized. The dried material was dissolved in 1 ml of 20 mM NaH2PO4:100 mM Na2SO4 (pH 6.8), and 200-µl aliquots were subjected sequentially to size exclusion HPLC on a BioSil TSK-125 column (7.5 x 300 nm; Bio-Rad, Mississauga, Ontario, Canada) equilibrated in the above phosphate buffer. Finally, the immunoreactive fractions (Chart 1B) from the separate TSK-125 column runs were pooled (2.5 ml), lyophilized, redissolved in 500 µl of 10 mM KH2PO4, pH 7.0, and injected onto a reverse-phase column (RP-P, 4.1 x 250 mm, 300 Å pore; Synchrom, Inc.) equilibrated with 10 mM KH2PO4 and eluted with a gradient of n-propyl alcohol (Chart 1C). The protein eluting at 35.5 min in 2.0 ml was recovered by lyophilization.

Amino acid analyses were performed after hydrolysis for 24, 48, and 72 hr in 6 N HCl (Aristar; BDH, Mississauga, Ontario, Canada) at 100°C in the laboratory of Dr. Yaguchi (Division of Biological Sciences, National Research Council, Ottawa, Canada) using a one-column procedure on a Durrum D-500 analyzer.

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**Chart 1.** Purification of immunoreactive material from ASV-NRK solid tumors by HPLC methods. A, ion-exchange HPLC on AX300 at 1.0 ml/min. The hatched area in A indicates the immunoreactive fractions pooled and subjected to B, size exclusion HPLC on a TSK-125 column at 0.5 ml/min. The hatched area in B indicates immunoreactive fractions pooled and subjected to reverse-phase HPLC. C, calibration of TSK-125 column. Standards used were: 1, bovine serum albumin (M, 68,000); 2, ovalbumin (M, 45,000); 3, chymotrypsinogen A (M, 25,000); 4, myoglobin (M, 17,500); 5, cytochrome c (M, 12,500); 6, aprotinin (M, 6,800); 7, insulin chain B (M, 3,500); and 8, insulin chain A (M, 2,500). x, retention time of rat hepatoma oncomodulin (M, 11,500) running anomalously at 18 min; f, retention time of immunoreactive material from ASV-NRK tumors. D, reverse-phase HPLC on RP-P at 0.8 ml/min. Material from the single peak eluting at 35.5 min was collected, lyophilized, and subjected to amino acid analysis (see Table 2).
RESULTS AND DISCUSSION

We have isolated and purified oncomodulin previously from Morris hepatoma 5123tc (10) and have detected its presence in 10 different rat hepatomas induced by a variety of chemical carcinogens (9, 11, 13, 15). The presence of oncomodulin in rat hepatoma has been confirmed recently by other investigators (4). These observations prompted us to investigate whether oncomodulin was also present in virally transformed cells and the tumors they produce. Material binding to anti-rat oncomodulin IgG was localized in ASV-transformed NRK cells by the indirect immunofluorescence technique (Fig. 1). Specific immunofluorescence was detected in the nucleus and, to a lesser extent, the cytoplasm of all cells in a logarithmically growing ASV-NRK population (Fig. 1B). No specific immunofluorescence was observed (i.e., was similar to that found with preimmune rabbit serum; Fig. 1C) when antiserum preadsorbed with rat hepatoma oncomodulin was used, indicating that the observed fluorescence was probably due to oncomodulin. Thus, oncomodulin appears to be localized mainly in the nucleus of cultured ASV-NRK cells. It occurred in all cells, and thus, the expression of oncomodulin was not a random event in a subpopulation of cells. By contrast, no specific immunofluorescence was detectable in uninfected, nonneoplastic NRK cells (Fig. 1, E and F) grown under identical culture conditions.

While these observations point to the presence of oncomodulin in NRK cells transformed by ASV, the question remained as to whether anti-rat hepatoma oncomodulin IgG was recognizing the same molecule as was isolated previously from rat hepatoma 5123tc. This question could be answered only upon isolating and characterizing the immunoreactive material present in ASV-NRK cells. To do this, the immunoreactive material in extracts of ASV-NRK solid tumors carried as xenografts in athymic mice was purified to homogeneity by a novel procedure using ion-exchange, size-exclusion, and reverse-phase HPLC (Chart 1). The purification is summarized in Table 1. Following ion-exchange HPLC, the immunoreactive material occurred only as a single peak coeluting with authentic rat hepatoma oncomodulin between 43 and 50 min (Chart 1A). These fractions were pooled, lyophilized, and fractionated further by size-exclusion HPLC (Chart 1B). Again, the immunoreactive material occurred only as a single peak coeluting with authentic rat hepatoma oncomodulin at 18 min (Chart 1B, A and C). From previous work (9–11), and from the amino acid sequence, it is known that the molecular weight of oncomodulin is 11,700. The elution time on size-exclusion HPLC of 18 min (equivalent to apparent M, 11,700) is essentially homogenous, with the major peak eluting at 35.5 min (Table 1, Chart 1C). The reason for this early elution is unknown, but it should be emphasized that the immunoreactive material from the B77-NRK tumors was exactly the same apparent molecular weight as authentic rat hepatoma oncomodulin. This material from size-exclusion HPLC was pooled and subjected to reverse-phase HPLC (Chart 1D). It was essentially homogenous, with the major peak eluting at 35.5 min, exactly the retention time of rat hepatoma oncomodulin. This material eluting at 35.5 min was shown to be immunoreactive (not shown). Finally, amino acid analysis of this material from reverse-phase HPLC revealed the composition to be identical to that of oncomodulin from rat hepatoma 5123tc (Table 2). Thus, virally transformed ASV-NRK cells contain the same oncomodulin molecule as that found in chemically transformed rat hepatoma 5123tc.

Specific radioimmunoassay indicated that nonneoplastic NRK cells in culture do not contain oncomodulin at levels above the limits of detection (2 ng/mg protein) of the assay (Table 3). However, viral transformation of these cells by ASV caused cellular oncomodulin levels to increase by at least 600-fold to 1200 ng/mg protein. This high level persisted when ASV-NRK cells were carried as solid tumors in athymic mice (Table 3),...
making these cells as rich a source of oncomodulin as rat hepatoma (11, 13, 15). In addition, 2 neoplastic transformants which arose "spontaneously" upon repeated passage of NRK cultures were cloned and found to contain lower, but measurable, levels of oncomodulin whether carried as solid tumors or in tissue culture (Table 3).

We conclude from these observations that oncomodulin can be detected in both virally and spontaneously transformed NRK cells, as well as in chemically induced rat liver tumors (9–11, 13, 15). Thus, it would appear that the means by which cells are transformed does not determine oncomodulin production. Although the cellular function of oncomodulin remains unknown, it is clearly produced as a direct consequence of neoplastic transformation. The levels of oncomodulin in transformed cells approach that of calmodulin (4, 13, 15), the ubiquitous calcium-binding protein implicated in the regulation of normal cell proliferation (2–5). The fact that cellular calmodulin levels also increase dramatically upon neoplastic transformation (4, 8, 14–17) suggests that changes in the total calcium-binding activity of the cell may be important to the expression of the transformed phenotype.

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Fig. 1. Immunological localization of immunoreactive material in NRK cells transformed by ASV. Nonneoplastic NRK cells and ASV-NRK cells were treated with antioncomodulin IgG or preimmune IgG and then with fluorescein isothiocyanate-conjugated anti-rabbit IgG as described in "Materials and Methods." ASV-NRK (A) and NRK (D) cells under phase contrast; ASV-NRK (B) and NRK (E) cells treated with rabbit anti-rat hepatoma oncomodulin IgG and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG; and ASV-NRK (C) and NRK (F) cells treated with preimmune rabbit IgG and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG.
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