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Biochemical Characterization of Transformation-Specific Proteins of Acute Avian Leukemia and Sarcoma Viruses

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The biological and biochemical properties of the transformation-specific proteins of three avian oncornaviruses with different oncogenic potentials were compared, namely the *gag-myc* protein of the avian myelocytomatosis virus MC29, the *gag-erb A* protein of the avian erythroblastosis virus AEV, and the *gag-fps* protein of Fujinami sarcoma virus FSV. These oncogenes were analyzed in transformed fibroblasts that expressed only the transforming proteins but showed no virus replication. Monoclonal antibodies against the viral structural protein p19, which is the N-terminus of the proteins, were used for indirect immunofluorescence, for immunoprecipitation of the proteins from subcellular fractions, and for immunoaffinity column chromatography. With this last method a 3000-fold purification of the proteins was obtained. By indirect immunofluorescence it was shown that the *gag-myc* protein was located in the nucleus, and bound to DNA after purification. The *gag-erb A* protein was not nuclear but probably located in the cytoplasm and did not bind to DNA after purification. Neither of the two proteins exhibited protein kinase activity. In contrast, the *gag-fps* protein did not bind to DNA but showed protein kinase activity after purification. It was not located in the nucleus either.

Key words: localization, purification of transforming proteins, avian viral oncogenes, nuclear antigen

Defective avian oncornaviruses transform cells by oncogenes that are generally fused to the viral structural *gag*-genes on the expense of other genes required for viral replication. These acutely transforming defective viruses code for polyproteins which in several cases consist of the structural protein p19 as N-terminus, which is part of the *gag*-gene, and a covalently linked transformation-specific protein [1,2]. Depending on the disease they caused, the viruses were classified as acute leukemia viruses, which predominantly induce leukemias, and sarcoma viruses, which give rise to solid tumors such as carcinomas or sarcomas [3]. Three virus isolates representative of three classes were chosen here for a more detailed and comparative analysis: the

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myelocytomatosis virus MC29 and the avian erythroblastosis virus AEV, both transforming bone marrow cells in vivo from different differentiation lineages as well as fibroblasts in vitro, and the Fujinami sarcoma virus, FSV, which causes sarcomas and also transforms fibroblasts in vitro [3,4]. The transformation-specific *gag*-linked fusion proteins of these three virus strains, designated as p110$^{gag\text{-}myc}$ [5], p75$^{gag\text{-}erbA}$ [6], and p130$^{gag\text{-}fps}$ [7,8], respectively, were analyzed for their cellular localization and biochemical properties after 3000-fold purification. These polyproteins were analyzed in comparison to the *gag*-fused polyproteins of replicating viruses that are not directly involved in transformation. For this approach, in vitro transformed nonproducer fibroblasts, which expressed the transformation-specific proteins only, and no viral structural proteins were used. The transformation-specific proteins were analyzed by monoclonal antibodies directed against the N-terminus of the structural protein p19 [9]. They were used for indirect fluorescence, immunoprecipitation, and purification by immunoaffinity column chromatography [10].

So far oncogenic transformation by avian retroviral oncogenes has been attributed to a plasma-membrane associated protein kinase [11]. However, this report shows that other mechanisms, involving for example, the nucleus and protein–DNA interaction, also may be involved in oncogenic transformation by retroviruses.

MATERIALS AND METHODS

Cells and Viruses

MC29-Q8-NP is an established nonproducer quail fibroblast cell line transformed by MC29 [12]. AEV-c1 23 is an AEV-transformed nonproducer chicken fibroblastic cell line supplied by Dr. S. Martin (Berkeley, CA). FSV-3Y1 c1 9 is a transformed FSV-rat nonproducer cell line subcloned in this laboratory, which originated from Dr. H. Hanafusa (New York, NY). The cells were grown in Dulbecco modified Eagle Medium (DMEM), 10 mM Hepes, 5% calf serum, 1% heat-inactivated chicken serum, and 0.5% DMSO.

Radioactive Labeling

Cells were grown in 10-cm petri dishes near to confluency, starved in medium without methionine for 2–4 hr, and labeled in methionine-free medium supplemented with 250–500 μCi/ml of ^{35}S-methionine for 2–4 hr. After washing, the cells were processed for further analysis or stored frozen at -70°C. Immunoprecipitation, immunoaffinity column chromatography, and immunofluorescence have been described previously [10].

RESULTS

Three fibroblast cell lines transformed by MC29, AEV, and FSV, respectively, were analyzed for localization of their *gag-onc* fusion proteins by using monoclonal antibodies against p19, which is the N-terminus of the proteins. Indirect immunofluorescence was performed with fluorescein-labeled antimouse antibodies, and we attempted to localize cellular substructures in two of the three cell lines by Nomarski optical differential interference microscopy. The result is shown in Figure 1. MC29-Q8-NP cells showed a strong nuclear fluorescence that excluded the nucleoli. The AEV-transformed cells show a diffuse staining probably of cytoplasmic origin. FSV-

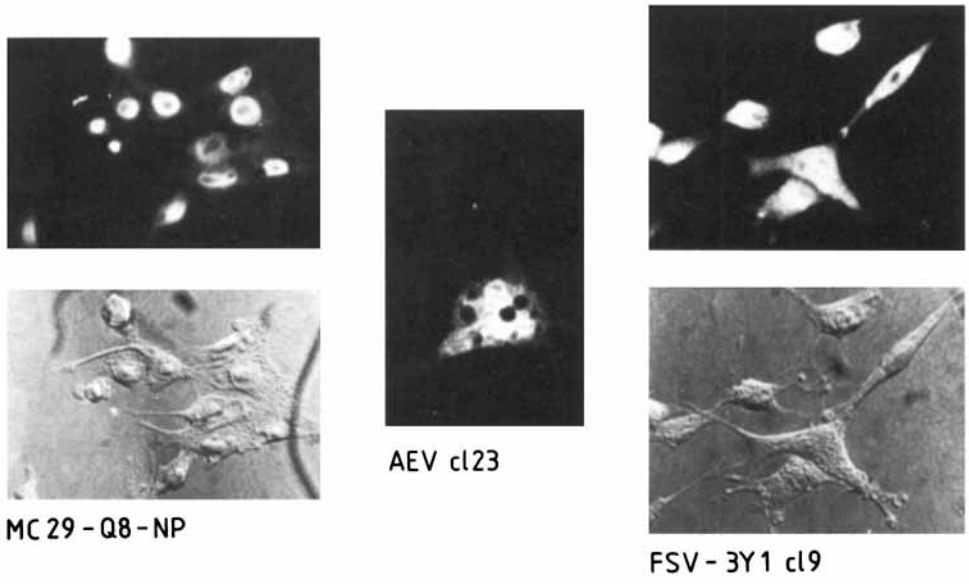

Fig. 1. Indirect immunofluorescence analysis of MC29-Q8-NP transformed quail fibroblasts, AEV cl 23 transformed chicken fibroblasts, and FSV-3Y1 cl 9 transformed rat cells. Indirect immunofluorescence was performed using monoclonal anti-p19 antibodies and FITC-labeled antimouse IgG antisera. In parallel, Nomarski interference optic analysis is shown except for AEV cells. ×1200.

transformed rat cells showed a cytoplasmic or membrane-associated fluorescence. Interestingly, an intensively fluorescing area at a cell-to-cell junction with the appearance of half a circle was detectable (upper right part). Furthermore, dots of cellular material left behind from retracted fibroblasts showed intensive fluorescent staining, especially at higher concentrations of antibodies. These properties of FSV-transformed cells are reminiscent of Rous sarcoma virus transformed chicken fibroblasts, where the protein kinase activity is found in the cytoplasmic membrane and adhesion plaques [13–15].

Purification of the Three p19-Containing Transformation-Specific Proteins

^{35}S-methionine-labeled fibroblasts, transformed with MC29, AEV, and FSV, respectively, were lysed and applied to an immunoaffinity column with monoclonal antibodies against p19. The column was washed extensively and subsequently the proteins were eluted with a buffer of pH 2. The eluted transformation-specific proteins were directly applied to a gel that was then analyzed by autoradiography. The purification was 3000-fold. Figure 2A shows the purified p110$^{gag\text{-}myc}$, the p75$^{gag\text{-}erbA}$, and the p130$^{gag\text{-}fps}$. Polyproteins containing p19 were purified from virus-producing but untransformed control cells, such as pr76^{gag}; 65K, 28K, and p19 are shown for comparison.

Interaction of Purified Transforming Proteins With DNA

The purified p110$^{gag\text{-}myc}$ protein was further analyzed for its ability to bind to DNA in a reconstituted reaction in vitro, since the immunofluorescence data (Fig. 1) indicated the accumulation of this protein in the nucleus. DNA-binding was assayed

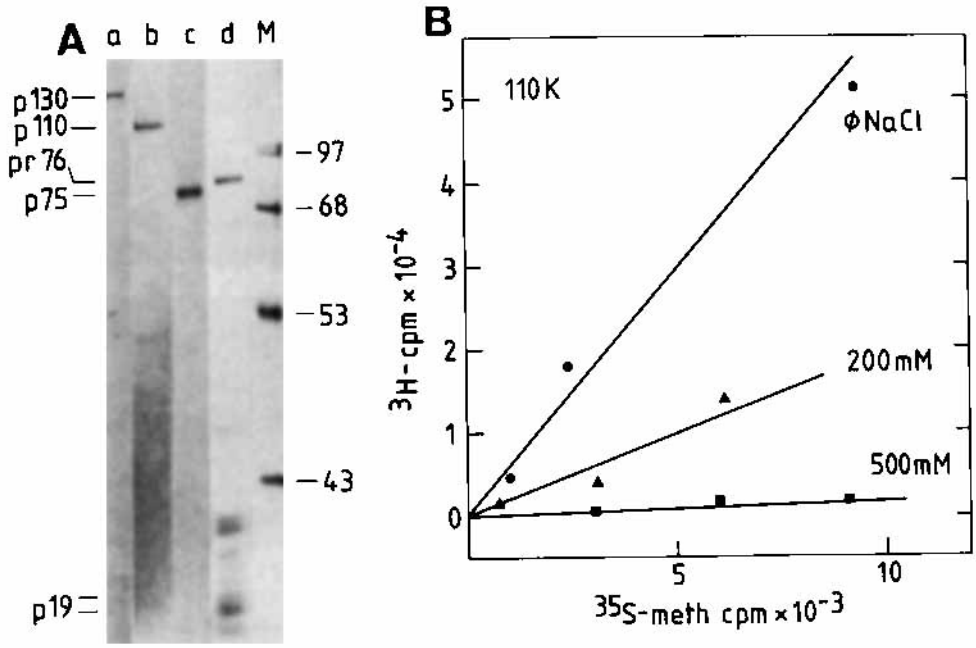

Fig. 2. (A). Polyacrylamide gel electrophoresis (10%) of the purified transforming proteins after elution from the immunoaffinity columns. Aliquots of the eluted peak fractions (50 μl out of 500 μl) were applied directly to the gel and exposed for autoradiography. (a) p130$^{gag\text{-}fps}$, (b) p110$^{gag\text{-}myc}$, (c) p75$^{gag\text{-}erbA}$, (d) Pr76^{gag}, and other *gag*-related proteins. (M) marker proteins. (B) Filter binding assay of the purified p110$^{gag\text{-}myc}$ protein (shown in A, slot b) with ^{3}H-thymidine-labeled chicken fibroblast DNA. The incubation mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8), 2 mM EDTA, 1-10 $\times$ 10^{3} cpm of ^{35}S-methionine-labeled p110$^{gag\text{-}myc}$ (ca 30,000 cpm/μg), and 2 μg of sheared DNA (ca 10 kb, 80,000 cpm/μg). NaCl was added to the incubation mixture at the final concentrations indicated. The ^{3}H-radioactivity retained on the nitrocellulose filters was determined by liquid scintillation counting.

in a filter-binding assay. ^{3}H-thymidine-labeled double-stranded DNA of about 10 kb and ^{35}S-methionine-labeled purified p110$^{gag\text{-}myc}$ were mixed and passed through nitrocellulose filters. As can be seen in Figure 2B, the protein retained DNA on the filter. The amount of DNA bound was linearly dependent on the protein input. The DNA-protein interaction was sensitive to the presence of salt ions. At a concentration of 200 mM NaCl the amount of DNA bound was about 50% reduced. No binding occurred at 500 mM salt (Fig. 2B). In contrast, the purified p75$^{gag\text{-}erbA}$, p130$^{gag\text{-}fps}$, and Pr76^{gag} proteins did not bind to DNA under the identical conditions (data not shown).

Protein Kinase Activity of the Purified Transforming Proteins

The most extensively analyzed avian sarcoma virus-transforming protein is pp60^{src} from the Schmidt–Ruppin strain D (SR-D) of Rous sarcoma viruses. Tumor-bearing rabbits induced by SR-D produce antisera (TBRS) that allow the protein kinase activity of the transforming protein to be detected in an immune-complex-bound phosphorylation reaction that phosphorylates the heavy chain of IgG (for review see [11]). Such a reaction is shown in Figure 3 using SR-D transformed

chicken embryo fibroblasts (CEF) and TBRS. In an analogous approach the fibroblast cell lines transformed with MC29, AEV, and FSV, respectively, were treated with antibodies against p19 produced in rabbits (Rαp19) or monoclonal antibodies ($\alpha p19^{mono}$). The immune complexes formed were tested for protein kinase activity. An immune complex-bound protein kinase activity was observed with FSV-transformed cells in agreement with previously published data [7,11]. Since the antibodies used were directed against p19 and not against the transformation-specific portions of the proteins, these reactions are different from the one performed with SR-D and

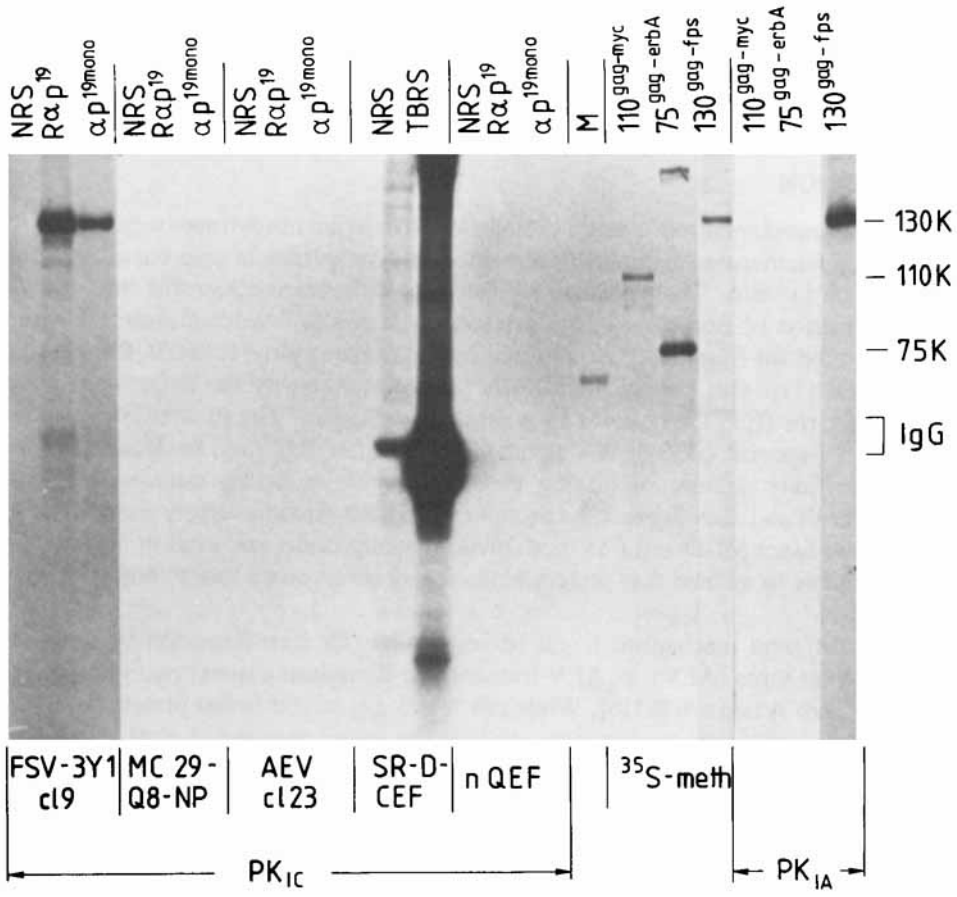

Fig. 3. Protein kinase assays of lysates of the transformed cell lines and control cells were performed using normal rabbit serum (NRS), rabbit anti-p19 serum (Rαp19), and anti-p19-monoclonal serum ($\alpha p19^{mono}$). As a positive control Schmidt–Ruppin D (SR-D) transformed chicken cells (CEF) were treated with tumor-bearing rabbit serum (TBRS). Normal quail (nQEF) served as negative control cells. Two kinds of protein kinase assays were performed. Left: Immunecomplex-bound reactions, indicated by PK_{IC}, involve immunoprecipitation of the transforming proteins from cellular lysates with antisera as indicated and addition of [γ-^{32}P]ATP to the precipitates for protein kinase reaction. The second assay, indicated by PK_{IA}, was performed with the purified transforming proteins still bound to the immunoaffinity column and incubation with [γ-^{32}P]ATP. All reaction products were subsequently applied to polyacrylamide gels and processed for autoradiography. The three transforming proteins served as markers (^{35}S-met); molecular weight markers (M).

TBRS. The transforming protein p130$^{gag\text{-}fps}$ itself becomes phosphorylated during this reaction [7]. No phosphorylation of p110$^{gag\text{-}myc}$ or p75$^{gag\text{-}erbA}$ or the IgG was detectable in the immune-complex-bound reactions with MC29 and AEV transformed cell lysates (Fig. 3, see PK_{IC}).

Since the immune-complex-bound phosphorylation reactions are very complex, we used the immunoaffinity-column-purified proteins to analyze whether at this stage of purification they exhibited protein kinase activity. To avoid artifacts or inactivation of the enzyme activity during elution from the immunoaffinity column, an aliquot of the immunoaffinity-column-bound material (which was extensively washed but not treated with elution buffer containing the 3000-fold purified protein) was analyzed for protein kinase activity by the addition of [γ-^{32}P]ATP (Fig. 3, see PK_{IA}). The p130$^{gag\text{-}fps}$ under these conditions showed a strong protein kinase activity that phosphorylated the molecule itself. No such activity was detected with the purified, immunoaffinity-column-bound p110$^{gag\text{-}myc}$ and p75$^{gag\text{-}erbA}$ proteins.

DISCUSSION

The transformation-specific proteins of three avian retroviruses were compared. The three retroviruses exhibit different oncogenic properties in vivo but all transform fibroblasts in vitro. These studies indicate that different mechanisms may result in transformation of fibroblasts. One mechanism seems to involve a protein kinase as was reported for fibroblasts transformed by the sarcoma virus Schmidt–Ruppin strain D (SR-D) [11]. Our results indicate that transformation by the defective Fujinami sarcoma virus (FSV) may occur by a similar mechanism. The protein kinase activity of the FSV-specific p130$^{gag\text{-}fps}$ was retained even after 3000-fold purification over an immunoaffinity column, indicating that the enzyme is closely associated with the protein itself and showing that the proteins from both sarcoma viruses may have more analogies. Since SR-D and FSV both predominantly cause sarcomas in the animal, it is reasonable to assume that protein kinases may be involved specifically in this type of disease.

A different mechanism might be responsible for transformation by avian erythroblastosis virus (AEV), as AEV-transformed fibroblasts express two virus-specific proteins, erb A and erb B [16]. While erb A is a *gag*-linked fusion protein that can be detected by antibodies against p19, erb B is not linked to structral viral proteins and cannot be studied until specific antibodies become available. Immunofluorescence analysis of AEV-transformed fibroblasts shown here indicate that erb A is probably a cytoplasmic protein. The immunoaffinity-column-purified protein was found not to have protein kinase activity nor did it bind to DNA. Yet the roles of both proteins in transformation or their possible interactions need further analysis.

In contrast, immunofluorescence analysis of MC29-transformed fibroblasts showed that the transformation-specific protein was located in the nucleus. Also, the immunoaffinity purified p110$^{gag\text{-}myc}$ protein interacted with DNA, most likely through the transformation-specific and not through the *gag*-specific region, since Pr76^{gag} and other *gag*-related polyproteins did not bind to DNA [10]. The p110$^{gag\text{-}myc}$ protein has some analogies to the transforming protein coded for by Simian virus 40 (SV40), the T-antigen, which is also a nuclear protein [17]. Whether the modes of DNA–protein interaction are also similar, needs further elucidation.

Since malignant transformation of fibroblasts or other cells most likely involves several steps, it remains to be seen whether the three described, apparently unrelated, transforming mechanisms ultimately result in a common event.

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