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TWO DIFFERENT VARIANTS OF THE SAME TROPOMYOSIN POLYPEPTIDE IN CLONES FROM GM1386 HUMAN SKIN FIBROBLASTS

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SUMMARY: A new protein observed in two-dimensional electrophoresis patterns of proteins from the human skin fibroblast line GM1386 has been identified as a charge and molecular-weight variant of the type of tropomyosin found in smooth muscle (Tm:3). This is the second variant of Tm:3 found in GM1386 and represents a second site mutation in one of the genes coding for Tm:3.

Human skin fibroblasts contain at least six proteins that have tropomyosin-like characteristics (1-3). These proteins each appear to be the product of a separate gene since each has a unique peptide cleavage pattern after limited proteolysis (3). In addition, separate DNA sequences have been identified for two of the six proteins (4) and charge variants (i.e., altered isoelectric point, suggesting an amino acid substitution) have been found in separate cell lines for Tm:3 (2; Cytosk:8 in ref.1) and Tm:4 (Cytosk:11 in ref. 1). In this paper we report the discovery of a second variant of Tm:3, named Tm:3.2, found in cultures of human fibroblast line GM1386 (normal skin), the same cell culture that expresses the original variant Tm:3.1 (2). Tm:3.2 is both a molecular-weight and charge variant of Tm:3 and was discovered after the parent cell line, GM1386, was cloned. The data indicate that Tm:3.2 is a biochemical variant of Tm:3, suggesting that one copy of the gene for Tm:3 has undergone mutation twice, resulting in the synthesis of two different altered gene products. These proteins are expressed by subpopulations of GM1386 fibroblasts in a mutually exclusive fashion.

MATERIALS AND METHODS

Cell culture and sample preparation. The parent cell lines GM1385 and GM1386 (human skin fibroblasts) were obtained from the American Type Culture

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Collection (ATCC) and from the NIGMS Human Genetic Mutant Cell Repository; fibroblast line 1494 was obtained from Meloy Laboratories (National Cancer Institute Contract No1-CP91000). Individual clones from GM1386 were isolated according to the method of Puck (5). Cells were maintained in culture and radiolabeled with [^{35}S methionine] as described previously (2). After radiolabeling, cells were either (A) solubilized with a solution containing 9 M urea, 4% Nonidet P40 (NP40), 2% (w/v) ampholytes (LKB pH 9-11), and 2% 2-mercaptoethanol; (B) precipitated with 95% ethanol for tropomyosin purification; or (C) extracted with 0.5% Nonidet P40 to prepare cytoskeletons as described previously (1,2).

Tropomyosin isolation. Tropomyosin was isolated from the parent line GM1386 and a clone of GM1386 (GM1386-7) according to the method described by Fine et al. (6). Approximately 0.5 g (wet weight) of human lymphoid cells (from a patient with chronic myelogenous leukemia) were used as a nonradioactive protein carrier to aid in precipitation of tropomyosin from the [^{35}S]methionine-labeled fibroblast preparations.

Two-dimensional electrophoresis. Isoelectric focusing was done as described by Anderson and Anderson (7) with LKB ampholytes (10% pH 2.5-4, 90% pH 3.5-10). Second-dimension sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done on linear 9-18% (w/v) polyacrylamide gels as described previously (8). Conditions of electrophoresis, staining, and destaining have been described (9). Dried gels were autoradiographed or fluorographed (10) on Kodak XAR-2 film.

Nitrocellulose transfer and staining. Fibroblast proteins were transferred from two-dimensional gels onto sheets of nitrocellulose (Schleicher and Schuell, Inc.) as described by Anderson et al. (11) and reacted with rat antichicken skeletal muscle tropomyosin serum (a gift from Dr. Alexander MacLeod, Ludwig Institute for Cancer Research, Cambridge, England) as previously described (2). The proteins with bound antibody were detected with peroxidase-conjugated rabbit antirat immunoglobulin G (Miles Laboratories) followed by reaction with horseradish peroxidase reagent (BioRad Laboratories) and hydrogen peroxide.

Peptide mapping by limited proteolysis. [^{35}S]-labeled protein spots were cored out of dried gels, digested with either chymotrypsin (50 µg/ml), *Staphlyococcus aureus* V8 protease (50 µg/ml), or trypsin (50 µg/ml) and the digestion products separated on 18% polyacrylamide gels as previously described (2). The final digestion products were detected by fluorography (10).

RESULTS

Figure 1 shows the two-dimensional electrophoresis pattern of proteins from fibroblast cell line 1494, the parent cell line GM1386, and from clones isolated from GM1386 (clones 1386-12 and 1386-7). The tropomyosin protein pattern shown for 1494 represents the pattern obtained for 97% of the 100 normal human fibroblast lines analyzed by our laboratory thus far, including samples of newborn foreskin. GM1386 has two proteins, Tm:3.1 and :3.2, not present in 1494. A majority of the GM1386 clones synthesized either the Tm:3.1 variant (105 of 143 clones) or a protein with similar isoelectric point

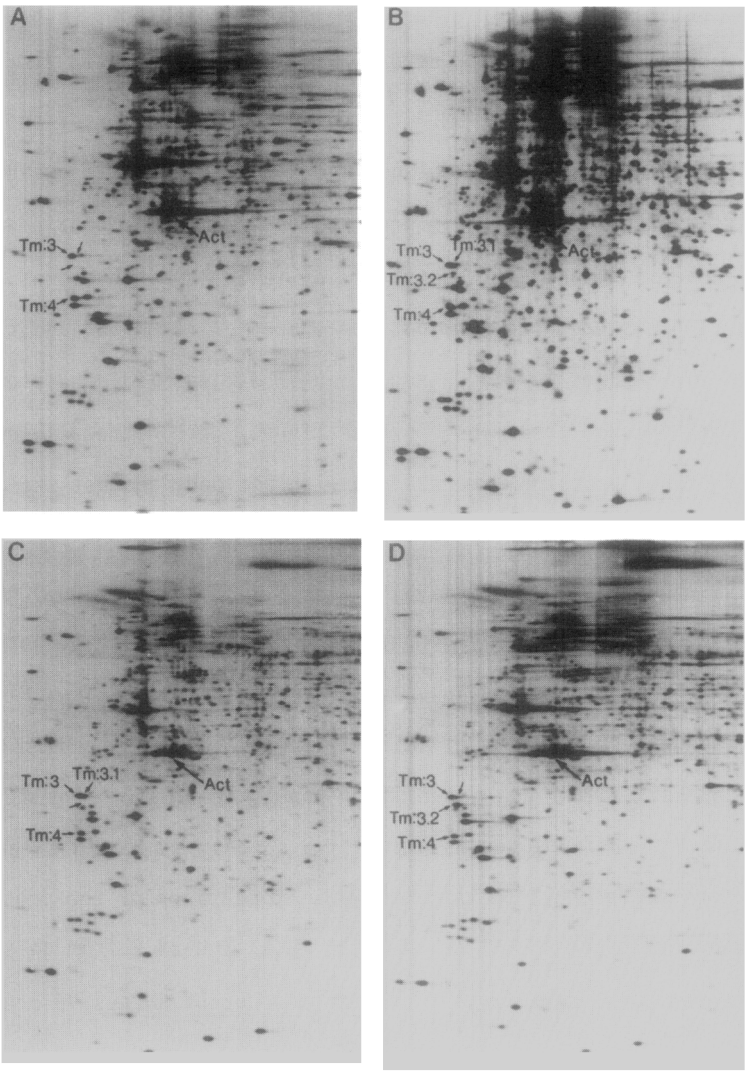


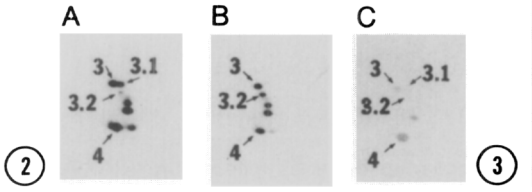
Figure 1. Two-dimensional electrophoresis patterns of total protein from human skin fibroblasts. (A) 1494; (B) GM1386; (C) GM1386, clone 12; (D) GM1386, clone 7. Act, actin; Tm, tropomyosin. Gels are oriented with the acidic side to the left and the basic side to the right.

but slightly lower molecular weight termed Tm:3.2 (30 of 143 clones). A small number of samples contained both Tm:3.1 and Tm:3.2 (8 of 143 clones). Similar results were obtained when GM1386 (passage 3) obtained directly from the NIGMS Human Genetic Mutant Cell Repository was cloned. The quantitative difference in the abundance of Tm:3.1 and :3.2 in the GM1386 parent line suggests that in the heterogeneous cultures cells expressing Tm:3.1 grow more efficiently than those expressing Tm:3.2. The morphology of the cells containing Tm:3.1 and

Tm:3.2 was identical to that of other normal skin fibroblasts (e.g., 1494). Both cell types were also found to have normal karyotypes and a finite life-span in culture. The cell line GM1385 (cells from the father of the GM1386 donor) also contains Tm:3.1 (2), but no Tm:3.2 was detected in two-dimensional electrophoresis patterns of protein preparations from cells obtained from the ATCC or the Genetic Mutant Cell Repository or in 10 isolated clones.

Tropomyosin was prepared from both the parent line GM1386 and the Tm:3.2 clone GM1386-7. Figures 2A and 2B show that Tm:3.2 was isolated along with Tm:3.1 and Tm:3 in such tropomyosin preparations. When nitrocellulose transfers of GM1386 proteins were stained with antibody against skeletal muscle tropomyosin, we detected Tm:3.2 in addition to Tm:3 and Tm:3.1, further verifying its identity as a tropomyosin (Fig. 2C). Cytoskeleton preparations from GM1386 and GM1386-7 clearly demonstrated the association of both Tm:3.1 and :3.2 with the detergent-insoluble cytoskeleton of the cells (Fig. 3).

The structural relatedness of Tm:3, :3.1, and :3.2 was studied by using limited proteolysis (12). Tm:4, the major nonmuscle tropomyosin of cultured fibroblasts (1,3), was included as a positive control since its cleavage pattern is very different from that of Tm:3. Comparison of the proteolytic fragments obtained after digestion of Tm:3, :3.1, and :3.2 from gels of GM1386 proteins with trypsin, chymotrypsin, or Staphlyococcus aureus V8 protease



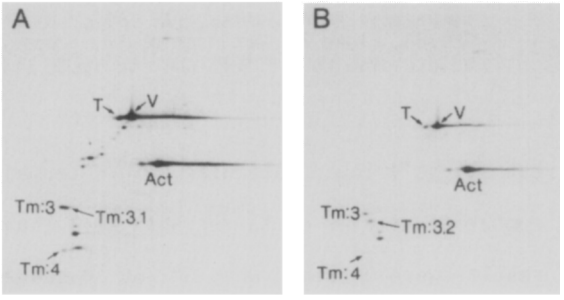


Figure 2. Identification of fibroblast tropomyosin. (A) Proteins present in tropomyosin preparation from GM1386; (B) proteins present in tropomyosin preparation from GM1386 clone 7; (C) proteins that reacted with antitropomyosin antiserum. Gel orientation and abbreviations are as in Fig. 1.

Figure 3. Two-dimensional electrophoresis patterns of cytoskeletal proteins isolated from (A)GM1386, clone 12 and (B)GM1386, clone 7. T, tubulin; V, vimentin. Other abbreviations and gel orientation are as in Fig. 1.

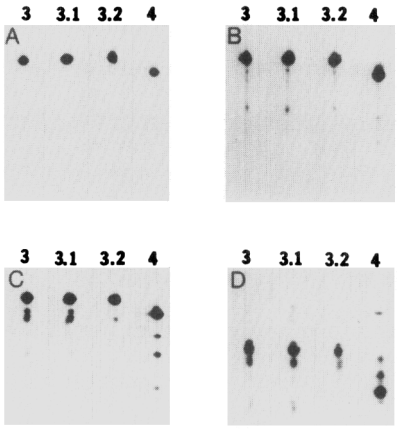


Figure 4. One-dimensional electrophoresis patterns of peptides after partial proteolysis with no protease (A), or with trypsin (B), chymotrypsin (C), or Staphylococcus V8 protease (D). The proteins studied in these experiments were Tm:3, Tm:3.1, Tm:3.2, and Tm:4.

suggested that these proteins have related, but not identical, amino acid sequences (Fig. 4). Although cleavage of Tm:3, :3.1, and :3.2 with trypsin produced seemingly identical patterns, after cleavage with chymotrypsin, Tm:3.2 lacked several of the high-molecular-weight peptides obtained from both Tm:3 and :3.1. Cleavage with V8 protease, on the other hand, produced more high-molecular-weight bands in the Tm:3.1 and :3.2 patterns than in the Tm:3 pattern. In contrast, the cleavage pattern of Tm:4 with all three enzymes was totally different from that of the Tm:3 or the Tm:3 variant proteins.

DISCUSSION

The human skin fibroblast cell lines GM1385 and GM1386 have previously been shown to contain a charge variant (Tm:3.1) of a protein described as smooth muscle tropomyosin (Tm:3; Ref. 2). Cloning experiments with both of these cell lines have now allowed separation of two different populations of cells from GM1386, one with the Tm:3.1 variant and a second with Tm:3.2. The expression of either Tm:3.1 (73%) or :3.2 (21%) but not both by a majority of the clones isolated from GM1386 provides evidence that the two proteins are products of different alleles at the same locus and hence are variants of the

same protein. The small percentage of clones that appeared to express both Tm:3.1 and :3.2 (6%) were probably impure, i.e., mixtures of Tm:3.1- and Tm:3.2-type cells. The cloning experiments verified that although Tm:3.1 is expressed by fibroblast line GM1385 (cells originally isolated from the father of GM1386 donor), Tm:3.2 is not. The expression of Tm:3.1 by cells originally isolated from a father and son indicates that Tm:3.1 is the result of a germ-line mutation, whereas the unique appearance of Tm:3.2 in the GM1386 cultured line suggests that Tm:3.2 is the result of a somatic mutation that occurred either in the donor (son) or early in culture (prior to passage 3).

Biochemical evidence indicates that Tm:3.2, like Tm:3.1, is a variant of the smooth muscle tropomyosin Tm:3. All three proteins are associated with the detergent insoluble-cytoskeleton, as would be expected of a microfilament-associated protein such as tropomyosin. All three proteins were enriched in tropomyosin preparations and reacted well with antitropomyosin antibodies. In addition, the peptides obtained after partial proteolysis of Tm:3, :3.1 and :3.2 with trypsin, chymotrypsin, or S. aureus V8 protease were very similar, indicating that the primary structures of these three proteins are closely related.

The appearance of Tm:3.2 is similar to the second-site alteration of β-actin in human fibroblasts that has been described by Leavitt et al. (13). The expression of two unique variants of β-actin by two different subpopulations of KD cells (13,14), suggested that two different sites on one of the genes coding for β-actin were mutated and, perhaps, that the first mutation predisposed that gene to be mutated again. Since the Tm:3 variants are expressed in a mutually exclusive manner, each in combination with the wild-type Tm:3, these proteins also most probably represent subsequent mutations in the same copy of the Tm:3 gene. The probability of such a double event occurring by chance (i.e., two spontaneous mutations in one gene within a few generations) is very low. Therefore, the first mutation of the Tm:3 gene appears to have predisposed it to subsequent mutation as in the case of β-actin in the KD cells. The occurrence of second-site mutational events in

two structural proteins such as actin and tropomyosin, assumed to be under rigid control due to their functional importance and evolutionary stability, is probably not a matter of coincidence. The cell lines expressing these variant proteins will be valuable tools in future investigations of the actin and tropomyosin genes.

ACKNOWLEDGMENT

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