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# Neuronal protein gene product 9.5 (IEF SSP 6104) is expressed in cultured human MRC-5 fibroblasts of normal origin and is strongly down-regulated in their SV40 transformed counterparts

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**Neuronal protein gene product 9.5 (PGP 9.5) most likely identical to ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) has been reported to be expressed almost exclusively in neuronal and neuroendocrine tissues. By two-dimensional (2D) immunoblotting, comigration and microsequencing of proteins recovered from 2D gels we have identified PGP 9.5/UCH-L1 as polypeptide IEF SSP 6104 ($M_r$ = 27000, pI = 5.49) in the comprehensive 2D gel cellular protein database of human embryonal lung MRC-5 fibroblasts [(1989) Electrophoresis 10, 76–115; (1990) Electrophoresis 11, 1072–1113]. This protein is expressed at high levels in quiescent and proliferating cultured normal fibroblasts and is strongly down-regulated (about 10 times) in their transformed counterparts.**

**Normal human fibroblast; SV40 transformed human fibroblast; PGP 9.5; UCH-L1; Transformation sensitive protein; 2D-gel electrophoresis**

## 1. INTRODUCTION

Two-dimensional gel electrophoresis is a powerful and widely used technique for separating the protein constituents of cultured cells or tissues [1,2]. In this way the 26 kDa protein gene product 9.5 (PGP 9.5) was found in human brain [3] in concentrations at least 20 times greater than in any other organ analyzed so far [3,4]. Immunoperoxidase labelling using rabbit anti-human PGP 9.5 serum further indicated that the expression of this protein is confined to neuronal and neuroendocrine tissue [5]. The primary structure of the protein has recently been obtained from amino acid sequencing of 50% of the protein and by sequencing a cDNA isolated from a human retinal library [6]. Most recently, the structure of the gene spanning 10 kb has been determined [7].

To date, studies of the expression of this protein have so far been performed on tissues and have not included cultured cells. By 2D immunoblotting, comigration and microsequencing of proteins recovered from 2D gels we have identified PGP 9.5 as polypeptide IEF SSP 6104 ($M_r$ = 27 000, p$I$ = 5.49) in the comprehensive 2D gel cellular protein database of human embryonal lung MRC-5 fibroblasts [8,9]. We show that this protein is expressed at high levels in quiescent and proliferating cultured normal fibroblasts and that it is strongly down-regulated in their SV40 transformed counterparts. The implications of our results are discussed in view of the recent finding that this protein may correspond to ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) [10].

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*Abbreviations:* 2D, two-dimensional; IEF, isoelectric focusing; SSP, sample protein number; PGP, protein gene product; UCH, ubiquitin carboxyl-terminal hydrolase

## 2. MATERIALS AND METHODS

### 2.1. *Cultured cells and tissues*

Normal (MRC-5) and SV40 transformed human MRC-5 fibroblasts (MRC-5 V2) [11] and normal and SV40 transformed keratinocytes (K14) [12] were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml). The brain was dissected from a 4-month normal human male fetus and used as a source for protein microsequencing. These experiments have been approved by the Ethical Scientific Committee of the Aarhus Amtskommune.

### 2.2. *Labelling of cells with [$^{35}$S]methionine*

Cells grown in microtiter wells (NUNC, Denmark) were labelled for 14 h with 0.1 ml of laboratory-made Dulbecco's modified Eagle's medium (1 g/l, $NaHCO_3$) lacking methionine and containing 10% dialyzed fetal calf serum and 100 μCi of [$^{35}$S]methionine (SJ204, Amersham) [13].

### 2.3. *2D gels, immunoblotting, immunofluorescence and silver staining*

The procedures for running 2D gels, immunoblotting, immunofluorescence, and silver staining have been previously described in detail [13–15]. Rabbit anti-human PGP 9.5 antiserum (RA95101) was obtained from UltraClone Ltd, UK.

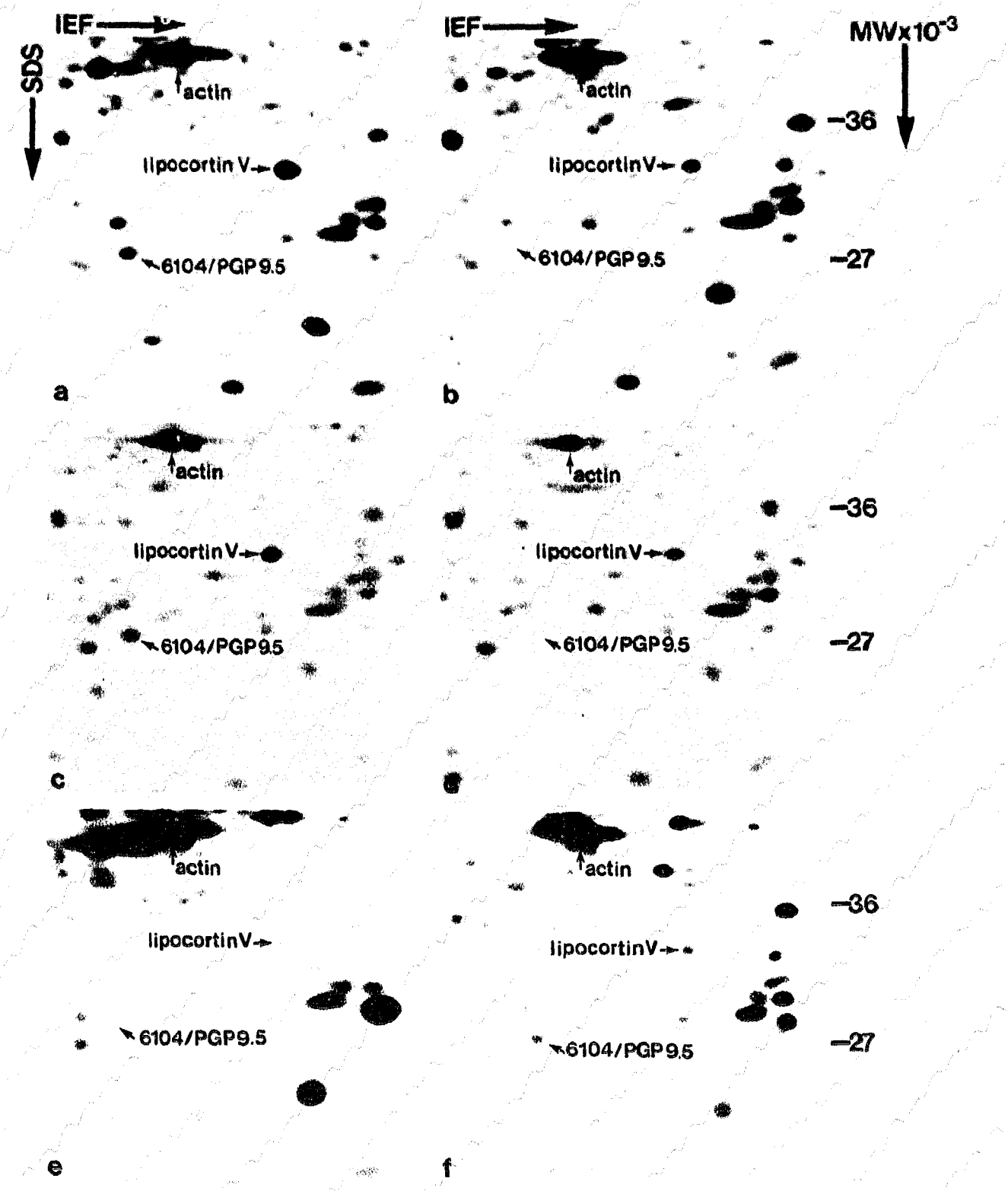


Fig. 1. Two-dimensional gel images (IEF) of polypeptides from pairs of normal and SV40 transformed cultured cells. (a, b) Fluorograms of [$^{35}$S]methionine labelled polypeptides from MRC-5 (a) and MRC-5 V2 (b) fibroblasts. (c,d ) Silver staining of MRC-5 (c) and MRC-5 V2 (d) proteins. (e, f) Fluorograms of [$^{35}$S]methionine labelled polypeptides from normal (e) and SV40 transformed (f) keratinocytes (K14). Only fractions of the gels are shown.

### 2.4. *Microsequencing*

Extracts of fetal human brain tissue were separated by 2D gel electrophoresis and stained with Coomassie brilliant blue. An aliquot of the [$^{35}$S]methionine labelled extract of human MRC-5 fibroblasts was added as a reference in order to match the Coomassie stained polypeptides relative to the [$^{35}$S]methionine labelled spots. The protein spot was cut from a number of gels and subjected to partial amino acid sequencing as previously described [16–18]. The sequences were compared with sequences in the Protein Identification Resources NBRF databank and the SWISS protein sequence bank.

## 3. RESULTS

Fig. 1 shows the [$^{35}$S]methionine-labelled protein 2D gel pattern (IEF) of normal human MRC-5 fibroblasts (Fig. 1a) and their SV40 transformed counterparts, MRC-5 V2 (Fig. 1b). Protein IEF SSP 6104 ($M_r$ = 27 000, p$I$ = 5.49) is synthesized in substantial amounts by cultured normal fibroblasts (Fig. 1a) and it is relatively abundant as estimated by silver staining (Fig. 1c). In contrast to the majority of other cellular MRC-5 polypeptides [9], the level of IEF SSP 6104 is down-regulated 10 times in the transformed cells relative to proliferating normal fibroblasts as determined by direct counting of the radioactivity contained in the spots (ratio MRC-5 V2/MRC-5 proliferating = 0.1). Normal quiescent and proliferating fibroblasts express this protein at approximately the same level (results not shown) [9].

2D gel analysis of proteins of other cultured cells of transformed origin such as HeLa and transformed human amnion cells (AMA) also showed extremely low levels of expression of IEF SSP 6104 [8,15]. This, however, is not a gereral pattern for transformed cells, since substantial levels are found in SV40 transformed keratinocytes (K14, Fig. 1f), while normal keratinocytes do not synthesize significant levels of this protein (Fig. 1e). *Xeroderma pigmentosum* cultured fibroblasts (XP25RO) [8] as well as cultured fetal fibroblasts from skin, ear, meninges and kidney (not shown) also express this protein at marked levels.

Comigration of [$^{35}$S]methionine labelled proteins from proliferating MRC-5 fibroblasts with protein extracts from fetal human brain indicated that this protein was very abundant in the brain (results not shown). To assess its identity several Coomassie brilliant blue spots recovered from 2D gels of brain extracts were subjected to microsequencing [16–18]. The protein was digested with trypsin and peptides wer separated by reversed-phase HPLC. Six peptides were analyzed: peptide 1, LGVAGQ; peptide 2, VYFMK; peptide 3, QTIGNSXG; peptide 4, LGFEDGSVLK; peptide 5, QFLSETEK and peptide 6, NEAIQAAHDAVAQEGQ. The sequences of these peptides matched perfectly with the sequence reported for the human protein gene product 9.5 (PGP 9.5): peptide 1, residues 20–25; peptide 2, residues 79–83; peptide 3, residues 84–91; peptide 4, residues 106–115; peptide 5, residues

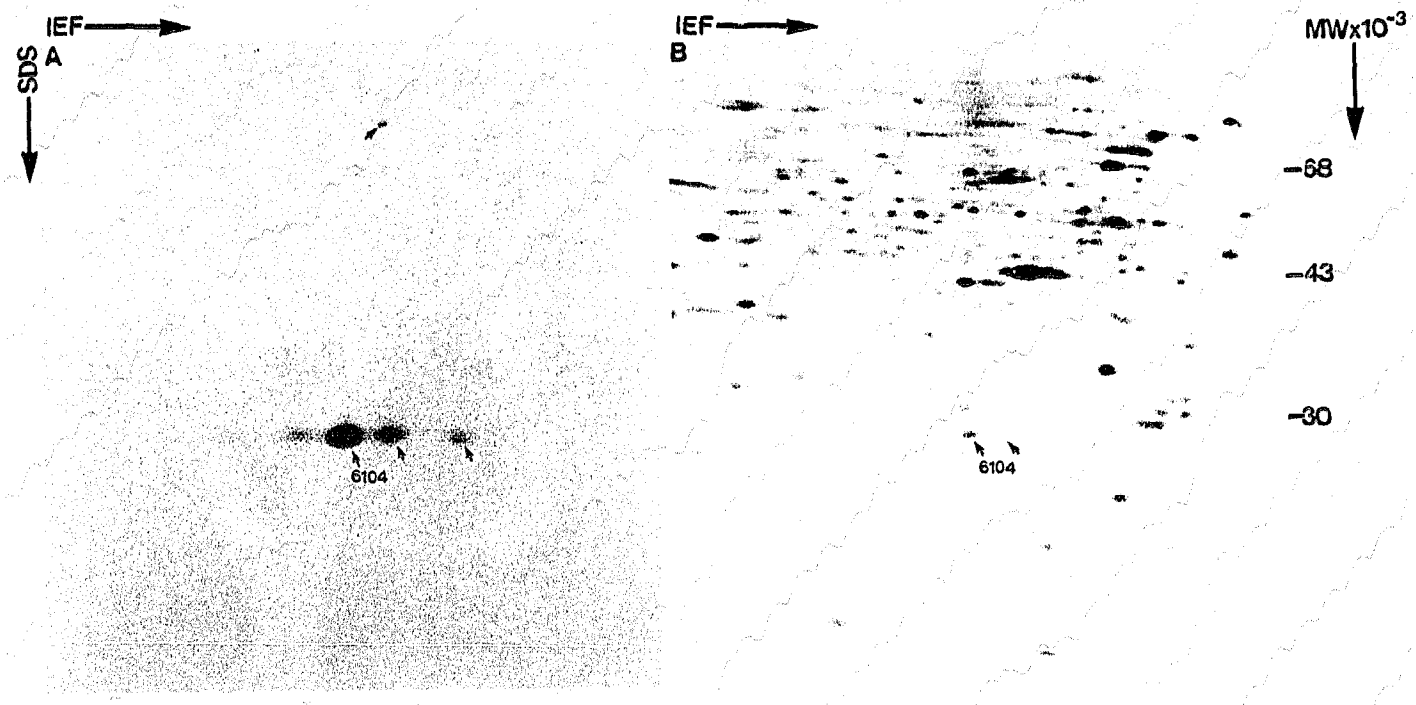


Fig. 2. 2D immunoblotting of MRC-5 proteins reacted with PGP 9.5 antibodies. (A) 2D immunoblotting. (B) Fluorogram used to localize the reacting spot.

116–123 and peptide 6, residues 136–151 as recently reported by Day et al. [7].

Further evidence indicating that IEF SSP 6104 corresponds to PGP 9.5 was obtained by 2D immunoblotting of MRC-5 proteins using rabbit polyclonal antibodies raised against PGP 9.5 (RA 95101, UltraClone Ltd, UK). As shown in Fig. 2, the antibodies react strongly with a 27 kDa protein (Fig. 2A) that corresponds to IEF SSP 6104 as judged by superimposing the X-ray fluorogram (Fig. 2B). The protein apparently consists of at least three charge variants. As judged by one-dimensional immunoblotting IEF SSP 6104/PGP 9.5 was barely detected in the transformed cells (compare Fig. 3A and B). Indirect immunofluorescence of methanol-fixed normal proliferating (Fig. 4a) and SV40 transformed fibroblasts (Fig. 4b) further confirmed the results obtained by immunoblotting as only MRC-5 cells reacted strongly with the antibodies. The reason for the Golgi staining observed in the transformed cells (Fig. 4b) is most likely due to cross-reacting proteins (arrows in Figs 2 and 3).

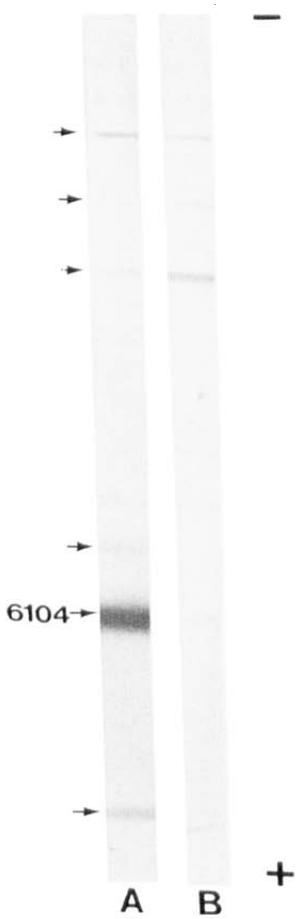


Fig. 3. One-dimensional blots of MRC-5 (A) and MRC-5 V2 proteins (B) reacted with PGP 9.5 antibodies. Proteins that cross-react with the antibodies are indicated with arrows.

## 4. DISCUSSION

Protein IEF SSP 6104 ($M_r$ = 27 000, p$I$ = 5.49) recorded in the comprehensive two-dimensional gel protein database of cellular proteins in human MRC-5 fibroblasts [9] was identified as protein gene product 9.5 (PGP 9.5) [6,7]. The identification was done by microsequencing of reversed-phase HPLC-purified tryptic peptides. We have sequenced six peptides amounting to 52 of the 223 amino acids reported to constitute the protein [7]. The six peptides were found to match perfectly with internal sequences in PGP 9.5. The identification was confirmed by 2D immunoblotting showing that polyclonal antibodies raised against PGP 9.5 react strongly with IEF SSP 6104. The presence of this protein in cultured normal fibroblasts, however, is somewhat surprising since PGP 9.5 has been reported to be a specific marker for neuronal and neuroendocrine tissue [5,19] and antibodies against the protein have been used in a number of immunochemical studies to stain tumours of neuronal and neuroendocrine origin [20–22] and innervation of organs [23–26]. One possible explanation for this discrepancy could be that the protein is down-regulated in fibroblasts in their normal environment in vivo and that culturing might induce its expression. We have largely confirmed the findings of Thompson et al. [5] that PGP

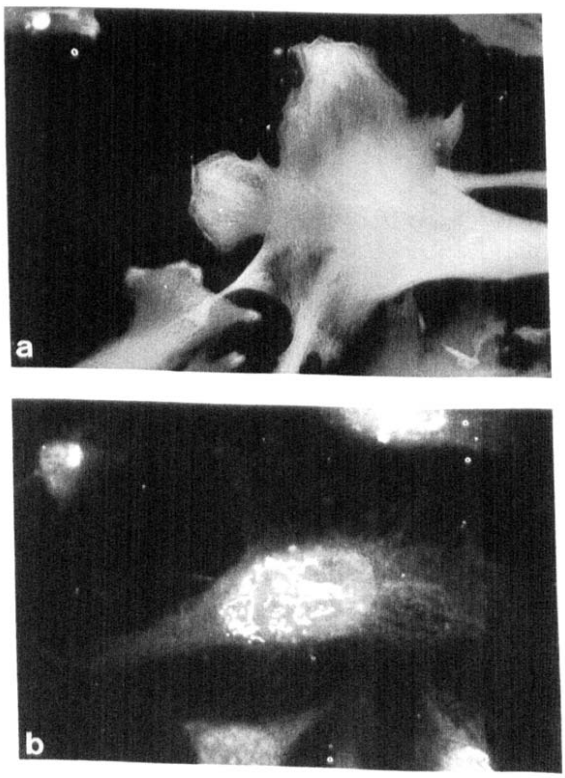


Fig. 4. Indirect immunofluorescence of methanol fixed normal (a) and SV40 transformed MRC-5 fibroblasts (b) reacted with PGP 9.5 antibodies.

9.5/IEF SSP 6104 is expressed in high amounts in neuronal tissue whereas the expression in most other tissues is low to undetectable (results not shown).

From bovine calf thymus a family of enzymes with ubiquitin carboxyl-terminal hydrolytic activity (enzymes hydrolyzing small molecule adducts of ubiquitin) have been isolated and defined lately [27]. Recent studies show that PGP 9.5/IEF SSP 6104 belongs to this family of ubiquitin carboxyl-terminal hydrolases (UCH) based on measurements of the enzymatic activity of purified bovine PGP 9.5 [10]. It is most likely that PGP 9.5/IEF SSP 6104 corresponds to ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) [10]. This is based on the similar physical and kinetic properties of the two proteins, UCH-L1 and PGP 9.5. Furthermore, the amino acid sequences of a number of purified tryptic peptides from bovine UCH-L1 and bovine PGP 9.5 are identical with the deduced amino acid sequence obtained from the cDNA sequence of human PGP 9.5 [10].

Ubiquitin is involved in a large variety of cellular processes [28,29]. The main one is likely to be the covalent attachment of several ubiquitin molecules to proteins thereby committing the conjugates to be degraded by specific proteases which release peptides and regenerate ubiquitin [29]. Other functions include the connection of a single ubiquitin molecule to histone molecules [30] probably playing a role in chromatin structure and gene expression [31]. Ubiquitin is also used to covalently modify receptors such as the T lymphocyte homing receptor [32], the platelet-derived growth factor receptor [33] and the growth hormone receptor [34].

The function of UCHs is unknown but may involve some of the above-mentioned processes, e.g. regulation of the specificity of the ubiquitin-dependent proteolysis system, control of gene transcription and cell cycle, regulation of the stress response, posttranslational processing of newly synthesized polyubiquitin and ubiquitin fusion proteins [27] and modification of receptor function [29]. Changes in the level of expression of PGP 9.5/IEF SSP 6104 (most likely identical to UCH-L1) in cells under various conditions, e.g. induced expression by culturing, down-regulated expression by SV40 transformation or otherwise, may thus affect a variety of functions in the cell.

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