Identification of M_r variants of prolactin with monoclonal antibodies

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Monoclonal antibodies (QB01 and 1200) prepared against human prolactin (hPRL) have helped define a variant form of the hormone. This variant is of apparently higher molecular mass (26 kDa) than the predominant form of the hormone (24 kDa) and its presence does not appear to be species-restricted. The demonstration of the 26 kDa form of hPRL in fresh pituitary tissue and amniotic fluid suggests it may retain some specific function.

Prolactin *Variant* *Fragment* *Monoclonal antibody* *SDS–PAGE* *Immunoblotting*

1. INTRODUCTION

Human prolactin (hPRL) is a single chain protein which shares extensive sequence homology (60–80%) with prolactins from other species [1]. Although it is phylogenetically related to both human growth hormone (hGH) and human chorionic somatomammotrophin (hCS), it retains only limited (15%) structural homology with these two hormones [2,3]. In addition to structural overlap, all 3 hormones display both lactogenic and somatotropic activities to varying degrees [4].

Growth hormone has been demonstrated to exist in a number of forms which include both structural variants derived from alternative processing of mRNA precursors [5] (20 kDa form), as well as variants which are believed to arise from post-translational modification of the intact molecule by proteases [6]. In the case of the former, it appears that alternative splicing of hGH nuclear RNA precursors results in a deletion of a linear sequence of 15 amino acids near the NH_2-terminal end of the molecule. Proteolytic modification hGH results in cleavage in the sequence region 134–146 [6] and gives rise to a 'two-chain' form composed of two polypeptides of 15 kDa and 7 kDa, respectively.

In addition to the predominant, 24 kDa form of prolactin [7,8], we have here identified a higher variant (26 kDa) as well as several fragments (18, 16 and 8 kDa) of the hormone with the aid of monoclonal antibodies (MAB). The 26 kDa form has been shown to be present also in purified bovine and ovine prolactin preparations as well as in crude human pituitary extracts and amniotic fluid. However, highly purified pituitary hPRL contained only the single species of monomeric hormone corresponding to 24 kDa. The demonstration of the 26 kDa variant form of hPRL in a number of 'native' preparations of the hormone is consistent with its possible physiological origin.

Abbreviations: hPRL, human prolactin; hGH, human growth hormone; hCS, human chorionic somatomammotrophin; MAB, monoclonal antibody

2. METHODS

2.1. *Preparation of hormones*

Human prolactin was affinity purified from the hPRL-rich side fractions derived during the

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purification of hGH from pituitary glands, based upon the method in [9] and obtained from the Public Health Lab. Service (Porton Down). This material was homogenised in sodium bicarbonate buffer (pH 8.3, 0.05 M) in the presence of phenylmethylsulphonyl fluoride (2 mM, PMSF) at 4°C, prior to two consecutive centrifugations of $1000 \times g$ and $20000 \times g$ in order to remove insoluble material. The supernatant was applied to an affinity column (20 × 1.2 cm) containing 100 mg monoclonal antibody (QB01) [10] immobilized on CNBr-activated Sepharose (Pharmacia). Retained material was washed extensively with phosphate-buffered saline (PBS) prior to elution with sodium iodide (2.5 mM) and dialysis against PBS. Highly purified hPRL was a gift from Dr P.J. Lowry (St. Bartholomews Hospital, London) whereas bovine PRL (bPRL) and ovine PRL (oPRL) were obtained from Dr M. Wallis (University of Sussex, Brighton). hGH was prepared as in [11] from individual human, cadaveric pituitary glands and stored frozen at −70°C. Thawed glands were homogenized in sodium bicarbonate buffer as above and solubilized in SDS-containing buffer [Tris–HCl, 100 mM (pH 6.8) containing SDS, 5% (w/v), dithiothreitol, DTT 100 mM and glycerol 10% (v/v)]. Similarly, human amniotic fluid, obtained freshly post-partum and stored at −20°C, was directly treated by boiling in SDS-buffer for 5 min.

2.2. *Monoclonal antibodies*

The antibodies derived from murine hybridoma cell lines have been prepared by standard techniques [12]. Antibody QB01 was highly specific for hPRL [10] and failed to cross-react with either hGH, hCS or with ovine and bovine prolactins. However, antibody 1200 [13] cross-reacted with ovine and bovine prolactins in addition to hPRL, as demonstrated here. Radioiodination of 1200 globulin (50 μg) was performed by the 'iodogen' method as in [14].

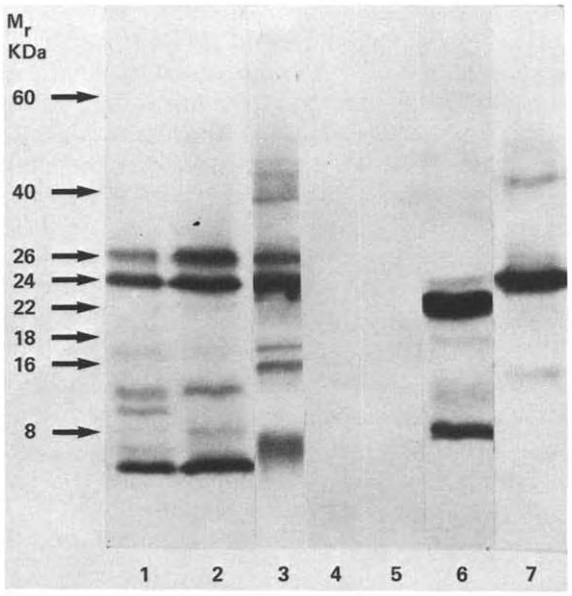
2.3. *SDS–PAGE and immunoblotting*

Polyacrylamide gel electrophoresis (PAGE) was performed as in [15] in the presence of SDS under reducing conditions. Solubilized proteins were applied to slab gels consisting of a 5% acrylamide stacking gel and a 15% running gel and subsequently submitted for immunoblotting. The electrophoretic transfer of proteins from SDS-gels to nitrocellulose sheets [16] was performed in Tris–glycine buffer (Tris 25 mM, glycine 20 mM, pH 8.2, containing 20%, v/v, methanol) for 2 h at 20°C. The nitrocellulose paper was subsequently immersed into 5% (w/v) bovine haemoglobin, 0.01% NaN_3 in PBS in order to block free binding sites on the paper. Radioiodinated antibody ([^{125}I]1200) was then added (20000 cpm/ml) to the nitrocellulose strips and shaken for 2 h at 22°C. This was followed by 3 consecutive washes with PBS (20 min/wash). Bands were visualized by autoradiography of the dried nitrocellulose sheets and molecular masses were determined from standard protein markers (BDH).

3. RESULTS

Examination of antibody QB01 affinity purified hPRL, by SDS–PAGE and immunoblotting with [^{125}I]1200 antibody, revealed the presence of two monomeric forms of the hormone with apparent molecular masses of 24 and 26 kDa, respectively (fig.1A, lanes 1,2). Dimeric prolactin was present in some preparations (lane 3) in much smaller quantities than the monomeric forms. Bands corresponding to fragments of hPRL were found despite the precaution of performing all steps at 4°C in the presence of PMSF. In the prolactin preparations shown in lanes 1 and 2, the fragments consistently ran with higher mobilities than the fragments for the preparation shown in lane 3. This difference probably arises from inadequate reduction of enzymatic activity in the tissue homogenates by the presence of PMSF and low temperature. Although the intensity and mobilities of these fragments were somewhat variable, three distinct bands corresponding to 18, 16 and 8 kDa, respectively, were discernable (lane 3). Such a band profile is consistent with the presence of two monomeric variant forms of hPRL (26 and 24 kDa), with the structural difference residing within only one fragment.

Binding of [^{125}I]1200 antibody to either hGH or hCS (fig.1A, lanes 4,5) was not observed, although the relative mobility of hGH to the prolactin bands is shown in lane 6. However, the only monomeric species of hPRL in preparations which had been highly purified by protein fractionation techniques



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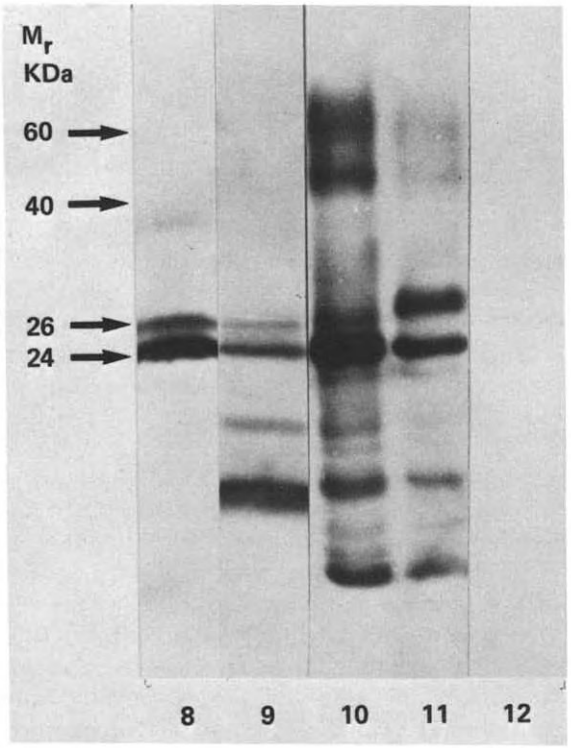

B

Fig.1. SDS–PAGE-immunoblot profile of prolactins developed with ^{125}I-labelled 1200 monoclonal antibody. (A) Lanes 1–3, different preparations of prolactin which have been affinity purified from human pituitary homogenate by monoclonal antibody QB01 (5 μg/lane); lane 4, hGH (10 μg); lane 5, hCS (10 μg); lane 6, [^{125}I]hGH; lane 7, hPRL highly purified by protein fractionation techniques [7] (10 μg). (B) Lane 8, SDS-solubilized human pituitary homogenate; lane 9, amniotic fluid 20 l; lane 10, bovine prolactin (10 μg); lane 11, ovine prolactin (10 μg); lane 12, SDS-solubilized rat pituitary homogenate.

[7] instead of by affinity chromatography, corresponded to the 24 kDa form (lane 7). Electrophoretic separation and immunoblotting of SDS solubilized preparations of human pituitary gland or of human amniotic fluid indicated that the 26 kDa variant was also present (fig.1B, lane 8 and 9, respectively). Fragments of hPRL were only identified in amniotic fluid preparations suggesting they may be 'normally' processed constituents of this fluid. The binding of [^{125}I]1200 antibody to a number of different fragments in affinity purified preparations derived from both pituitary glands and solubilized amniotic fluid indicates that the corresponding determinant may be repeated within the hormone's antigenic structure. An analogous situation exists for hGH, where it has recently been shown that one MAB can bind to two structurally non-overlapping fragments [17].

The cross-reactivity of [^{125}I]1200 with non-primate prolactins permitted the examination of 3 preparations for the presence of the 26 kDa variant form. Preparations of both bPRL (lane 10) and oPRL (lane 11) gave demonstrable quantities of the 26 kDa band; however, this was substantially reduced in the case of bPRL. Furthermore, preparations of rat pituitary glands which had been solubilized in SDS-buffer resulted in no binding, suggesting that this determinant is species-restricted (lane 12). Fragments of prolactin were furthermore observed in bPRL and oPRL preparations in addition to significant amounts of material of slower mobility (45–52 kDa).

4. DISCUSSION

Two monoclonal antibodies, one specific for

hPRL (QB01) and one also cross-reacting with certain non-primate prolactins (1200) have enabled the identification of a 26 kDa variant form of the hormone. Its demonstration in both pituitary gland and amniotic fluid preparations indicates that it is physiologically produced. It is highly unlikely that the 26 kDa variant is a prolactin-related protein with partial structural homology since the two antibodies employed bind to distinct determinants within the hormone structure. Furthermore, neither hGH or hCS cross-reacted with either of these monoclonal antibodies despite the phylogenic homology which exists between these hormones and prolactin. The major form of prolactin described exhibits an apparent molecular mass of 24 kDa on SDS–PAGE and represents the only identifiable monomeric species in preparations of the hormone derived by protein fractionation techniques [7,18]. This result suggests that the 26 and 24 kDa forms differ significantly in physical or chemical characteristics.

Several fragments of PRL have been observed, in addition to the 26 kDa variant in affinity purified preparations of the pituitary hormone and in amniotic fluid. Their variation in electrophoretic mobility and their absence from preparations of SDS-solubilized fresh pituitary tissue suggests that they may result from non-specific cleavage by proteases. However, some of these fragments (fig.1A, lane 3) displayed molecular mass profiles consistent with them being derived from two monomeric species differing in structure at either the N- or C-terminal ends of the molecule. An analogous system has been demonstrated with a genetic variant of hGH which retains a molecular mass of 20 instead of 22 kDa and results from a 15 amino acid deletion in the sequence of the latter form. Studies [19] have indicated that proteolytically cleaved forms of rat prolactin are biologically active and retain properties distinct from the parent molecule. Indeed, extensive studies with cleaved forms of hGH have shown that such modified forms have amplified biological activities [20].

Molecular heterogeneity of PRL preparations has been noted in [21] during the separation of 'isohormones' on a charge basis. However, these forms were apparently indistinguishable on the basis of molecular mass and were separated on polyacrylamide gels under non-denaturing conditions. Furthermore, multiple immunoactive components of 'large' forms of hPRL (40–50 kDa) have been identified in the plasma of patients with pituitary tumours [22]. However, authors in [23] have demonstrated that extraction of prolactin at acid pH can lead to polymerization of the hormone. Identification of high forms (40–50 kDa) of hPRL in purified or processed preparations of the hormone but not in pituitary or amniotic fluid raises the possibility that they are artifactual. The consistent demonstration of the 26 kDa variant form of prolactin in affinity purified hormone, pituitary and amniotic fluid preparations and some non-primate preparations of the hormone favours the view that it is physiologically produced. Although the underlying structural differences which result in the slower mobility of the 26 kDa form have not been established, it is conceivable that they stem from differences in amino acid sequence. Furthermore, the complete absence of the 26 kDa form from highly purified prolactin preparations indicates that it retains significant physiochemical differences from the major molecular species (24 kDa). Structural variation in prolactin molecules may have arisen from genetic polymorphism within a population. However, this seems unlikely since pituitaries obtained from at least 8 individuals were examined and demonstrated to contain both the 24 and 26 kDa variant forms of the hormone.

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