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Interspecies conservation of structure of interphotoreceptor retinoid-binding protein

Similarities and differences as adjudged by peptide mapping and *N*-terminal sequencing

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Structural properties of the retinal extracellular-matrix glycolipoprotein interphotoreceptor retinoid-binding protein (IRBP) from human, monkey and bovine retinas have been compared. SDS/polyacrylamide-gel-electrophoretic analysis of limited tryptic and *Staphylococcus aureus*-V8-proteinase digests show virtually identical patterns for the monkey and human proteins, whereas both sets differ considerably from the bovine protein pattern. Time-course digestion shows monkey IRBP to be more readily cleaved than bovine IRBP and also cleaved to smaller fragments. Also, reversed-phase h.p.l.c. of complete tryptic digests of the IRBPs indicate that, although they have in common a similar preponderance of hydrophobic peptides, all three proteins differ extensively in their fine structure. The *N*-terminal sequences of monkey and bovine IRBPs have been extended beyond those presented in our previous report [Redmond, Wiggert, Robey, Nguyen, Lewis, Lee & Chader (1985) Biochemistry **24**, 787–793] to over 30 residues each. The sequences yet show extensive homology, differing at only two positions, although the major monkey sequence has an additional five amino acid residues at its *N*-terminus ('*n*+5' sequence) not observed with bovine IRBP ('*n*' sequence). The newly determined *N*-terminal sequence of human IRBP demonstrates the presence of equal amounts of the '*n*' and '*n*+5' sequences that are qualitatively identical with those of the monkey. The presence of the five-amino-acid-residue extension in primate, but not bovine, IRBP may indicate variation in post-translational processing.

INTRODUCTION

Interphotoreceptor retinoid-binding protein (IRBP), first identified as the '7S protein' of the retina (Wiggert *et al.*, 1976), is a large glycolipoprotein found in the extracellular interphotoreceptor matrix between the neural retina and the retinal pigment epithelium cell layers and is the only retinoid-binding protein in this extracellular compartment (Adler & Klucznik, 1982; Pfeffer *et al.*, 1983; Bunt-Milam & Saari, 1984). IRBP is thought to mediate retinoid transport between the retinal photoreceptors and the retinal-pigment epithelium (Chader *et al.*, 1983), as evidenced by differential retinoid binding in light–dark experiments (Wiggert *et al.*, 1979; Lai *et al.*, 1982; Liou *et al.*, 1982; Adler & Evans, 1985). IRBP may also be involved in intercellular fatty acid transport, since it also binds a number of fatty acids, both covalently and non-covalently (Bazan *et al.*, 1985). In addition, or alternatively, the protein may serve a structural role, since it constitutes up to 70% of the readily soluble protein of the interphotoreceptor matrix (Pfeffer *et al.*, 1983). IRBP is a relatively hydrophobic asymmetrical protein of apparent M_r of about 140000 as assessed by SDS/polyacrylamide-gel electrophoresis, whether isolated from bovine (Fong *et al.*, 1984*a*; Adler *et al.*, 1985; Redmond *et al.*, 1985; Saari *et al.*, 1985), human (Fong *et al.*, 1984*b*) or monkey (Redmond *et al.*, 1985) retina. Monkey and bovine IRBPs also show significant homology in their *N*-terminal sequences (Redmond *et al.*, 1985). Partial cDNA clones for bovine IRBP have been reported (Barrett *et al.*, 1985).

In our study of the structure of IRBP, it is our ultimate aim to identify those parts of the molecule important in retinoid binding, fatty-acid binding and possible cell-surface interactions and, since the protein has recently been found to induce an intense immunological reaction in test animals (Gery *et al.*, 1986), to determine peptide sequences carrying immunological determinants. In the first phase of this study we now report on (1) peptide mapping of proteolytic digests of monkey, human and bovine IRBPs and (2) extended *N*-terminal sequences for bovine and monkey IRBPs as well as two separate, yet homologous, *N*-terminal sequences for human IRBP.

MATERIALS AND METHODS

Purification of bovine, human and monkey IRBPs

Bovine, human and monkey IRBPs were isolated and purified by using previously published methods (Redmond *et al.*, 1985). Bovine retinae were obtained frozen from Hormel (Austin, MN, U.S.A.). Monkey eyes were obtained from rhesus (*Macaca mulatta*) monkeys, 1–3

Abbreviations used: IRBP, interphotoreceptor retinoid-binding protein; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK'); TFA, trifluoroacetic acid.

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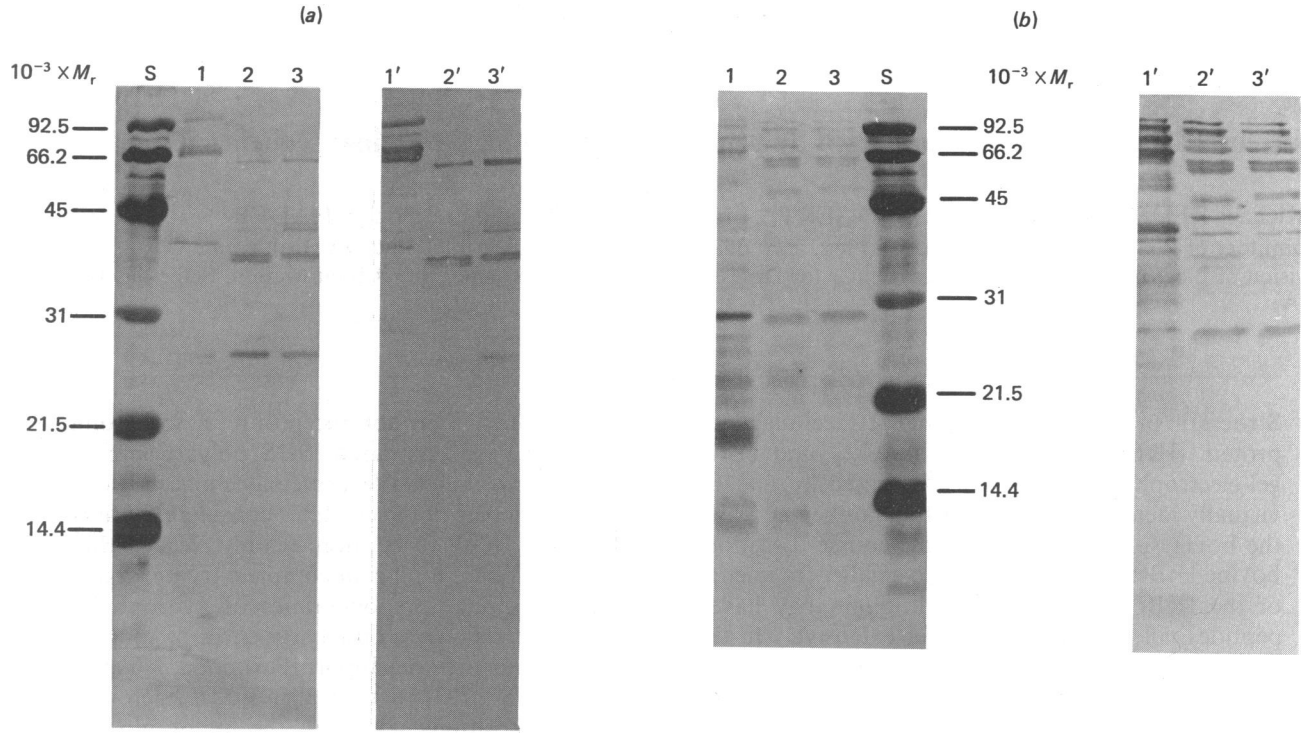

Fig. 1. Comparison of one-dimensional limited-proteolytic-digest peptide maps of bovine, human and monkey IRBPs

IRBP samples (50 μg) were digested for 30 min with either Tos-Phe-CH_2Cl-treated trypsin or *S. aureus* V8 proteinase (enzyme/substrate ratio 1:50) at 37 °C. The samples were divided equally between duplicate SDS/16%-(w/v)-polyacrylamide gels and electrophoresed. One duplicate gel was immunoblotted by using goat anti-(bovine IRBP) antiserum as the first antibody. (*a*) Tos-Phe-CH_2Cl-treated-trypsin digestion. S, M_r standards; 1 and 1′, bovine IRBP SDS/polyacrylamide-gel electrophoresis and immunoblot; 2 and 2′, human IRBP SDS/polyacrylamide-gel electrophoresis and blot; 3 and 3′, monkey IRBP SDS/polyacrylamide-gel electrophoresis and blot. (*b*) V8 proteinase digestion. S, M_r standards 1 and 1′, bovine IRBP SDS/polyacrylamide-gel electrophoresis and immunoblot; 2 and 2′, human IRBP SDS/polyacrylamide-gel electrophoresis and blot; 3 and 3′, monkey IRBP SDS/polaycrylamide-gel electrophoresis and blot.

years of age, exsanguinated under deep barbiturate anaesthesia. Human eyes were normal eye-bank donor eyes obtained through the National Diabetic Research Interchange (Philadelphia, PA, U.S.A.).

Limited proteolytic digestion: SDS/polyacrylamide-gel electrophoresis immunoblotting

Portions of bovine, monkey and human IRBPs dialysed against 0.1 M-NH_4HCO_3, pH 8.0, were digested with Tos-Phe-CH_2Cl-treated trypsin (Worthington, Freehold, NJ, U.S.A.) (enzyme/substrate ratio 1:50) for 15–120 min at 37 °C, as indicated in the relevant Figure legends. Proteolysis was stopped by the addition of (final concentrations in parentheses) 0.5 M-Tris/HCl, pH 6.8 (90 mM), 10% (w/v) SDS (2%) and β-mercaptoethanol (10%) with boiling at 100 °C for 2 min. In some experiments, portions of bovine IRBP were incubated for 24 h with 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M-all-*trans*-retinol (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the dark at 4 °C, with ethanol as control. The all-*trans*-retinol was dissolved in 100% ethanol. The final ethanol concentration did not exceed 2%. The samples were digested with Tos-Phe-CH_2Cl-treated trypsin for 15 min at 37 °C. Digestion was stopped as described above. The tryptic digests were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). For limited *Staphylococcus aureus*-V8-proteinase (Worthington, Freehold, NJ, U.S.A.) digestion (Cleveland *et al.*, 1977), portions of bovine, human and monkey IRBPs were mixed with (final concentrations in parentheses) 0.5 M-Tris/HCl (125 mM), 10% SDS (0.2%) and the *S. aureus* V8 proteinase (enzyme/substrate ratio 1:50) with incubation at 37 °C for 30 min and stopped by addition of 10% SDS and β-mercaptoethanol as described above. Duplicate SDS/polaycrylamide gels were run; one duplicate was stained with Coomassie Brilliant Blue or silver-stained (Bio-Rad, Rockville Center, NY, U.S.A.) and the other gel was immunoblotted (Towbin *et al.*, 1979). Primary antisera used were either goat anti-(bovine IRBP) serum or goat anti-(monkey IRBP) serum, as appropriate, at a dilution of 1:100, and using horseradish-peroxidase-conjugated rabbit anti-goat IgG–F(ab′)$_2$ fragments (Cappel Laboratories, Cochranville, PA, U.S.A.) as second antibody.

Reversed-phase h.p.l.c. peptide mapping

Proteins for reversed-phase h.p.l.c. peptide mapping were exhaustively dialysed against 0.1 M-NH_4HCO_3, pH 8.0, and concentrated against the same buffer in a Pro-Di-Con negative-pressure concentrator (Pierce Chemicals, Rockford, IL, U.S.A.). Approx. 1 nmol samples ($\sim$ 140 μg) of each of bovine, monkey and human IRBPs were digested with Tos-Phe-CH_2Cl-treated trypsin at an enzyme/substrate ratio of 1:50 at 37 °C for 24 h. The reaction was stopped by addition of 100% acetic acid, followed by freezing on solid CO_2 and

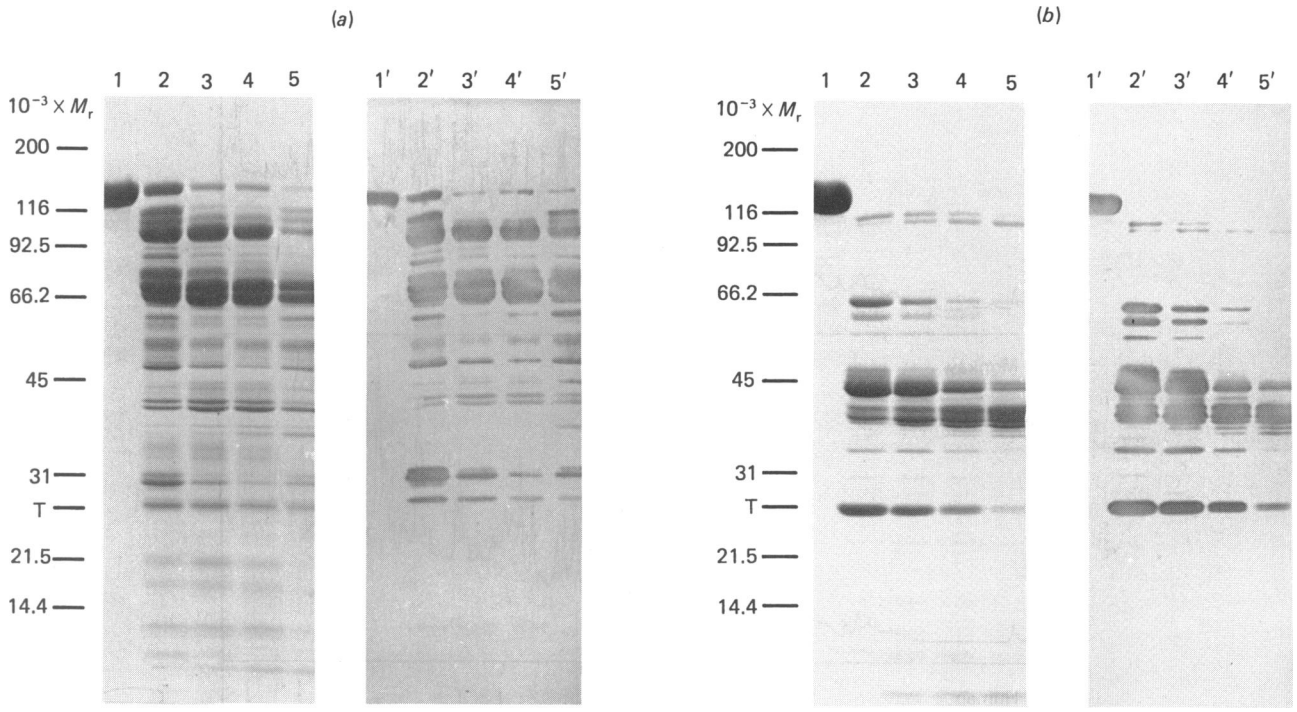

Fig. 2. Time-course analysis of tryptic digestion of bovine and monkey IRBPs

Aliquots (150 μg) of each protein were subjected to digestion by Tos-Phe-CH_2Cl-treated trypsin for 0, 15, 30, 60 and 120 min at 37 °C. The digests were resolved on SDS/8–20%-(w/v)-gradient-polyacrylamide gels. Two duplicate gels were run. One was stained with Coomassie Brilliant Blue; the other was immunoblotted using goat anti-(bovine IRBP) antiserum for the bovine blot and goat anti-(monkey IRBP) antiserum for the monkey blot as the primary antibodies. (*a*) Bovine; (*b*) monkey. 1, 0 min; 2, 15 min; 3, 30 min; 4, 60 min; and 5, 120 min. 1′–5′ are the corresponding lanes on the immunoblot. M_r values are shown on the left-hand side of each set. T refers to the M_r of trypsin.

freeze-drying. For h.p.l.c. analysis, each sample was redissolved in aq. 0.05% TFA. Samples were applied to a Zorbax Gold series C8 column (du Pont, Wilmington, DE, U.S.A.) in a Waters (Milford, MA, U.S.A.) gradient h.p.l.c. system. Elution was with aq. 0.05% TFA to 0.05% TFA in aq. 70% acetonitrile with simultaneous detection at 210 and 280 nm. Alternatively, samples were applied to a Waters μBondapak C_{18} column in a Beckman–Altex gradient h.p.l.c. system. Elution was with a gradient of aq. 0.05% TFA to 0.05% TFA in 80% propan-2-ol with detection at either 216 or 220 nm.

N-Terminal sequencing

Samples for *N*-terminal sequencing were dialysed exhaustively against 0.1 M-NH_4HCO_3, pH 8.0, with or without the addition of 0.05% SDS. The dialysed samples were then freeze-dried. Samples were sequenced by Applied Biosystems (Foster City, CA, U.S.A.) on an Applied Biosystems 470A gas-phase sequenator (Hewick *et al.*, 1981). The resultant amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. (Hunkapiller & Hood, 1983).

RESULTS

Comparison of limited proteolytic digests of human, monkey and bovine IRBPs

SDS/polyacrylamide-gel-electrophoretic analysis of limited tryptic digests of human, monkey and bovine IRBPs showed near identity between the tryptic-fragment patterns of the human and monkey IRBPs and a relative lack of identity of the two primate IRBPs with the bovine IRBP (Fig. 1*a*). Immunoblot analysis indicated that, with all three species, the smallest detectable immunoreactive fragment, using goat anti-bovine IRBP, had an M_r of about 25000. In addition, SDS/polyacrylamide-gel-electrophoretic analysis of limited *S. aureus*-V8-proteinase digests of bovine, human and monkey IRBP (Fig. 1*b*) indicated a similar finding, confirming the closer identity of the human and monkey proteins. As with trypsin digestion, the smallest immunoreactive *S. aureus*-V8-proteinase fragments on immunoblot analysis had, for all three proteins, an M_r of about 25000; these fragments, however, were not necessarily the same.

Time-course digestion of bovine and monkey IRBPs

A kinetic study of the tryptic digestion of bovine and monkey IRBPs (Fig. 2) showed further evidence of the distinctiveness of the monkey and bovine patterns. In both of the patterns, there are five or six major fragments. The monkey IRBP, however, appears to be more rapidly cleaved to lower-M_r fragments than does the bovine protein. The major fragments of the bovine IRBP at 30 and 60 min of digestion correspond to M_r values of 98000, 70000 and 67000 (Fig. 2*a*). The monkey IRBP after 30 and 60 min of digestion shows major fragments of M_r 63000, 43000, 38000–40000 and 25000 (Fig. 2*b*).

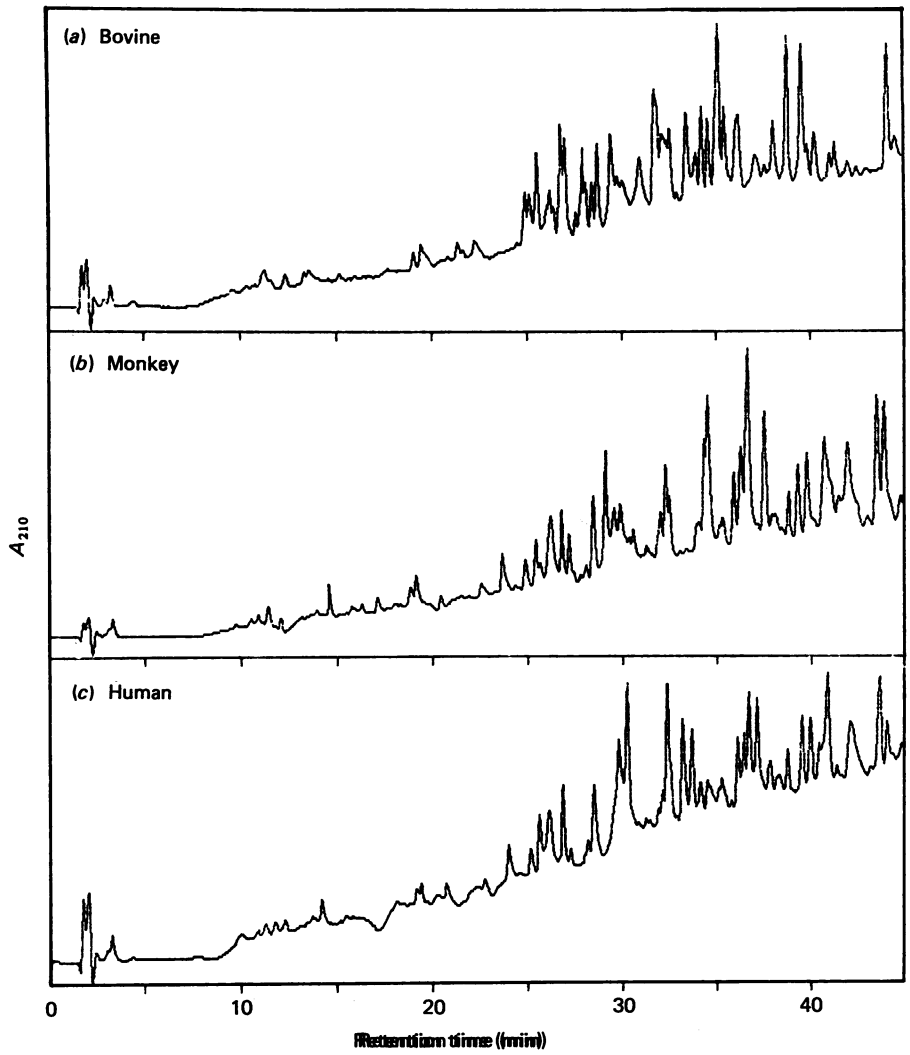

Fig. 3. Comparison of reversed-phase h.p.l.c. tryptic peptide maps of bovine, monkey and human IRBPs

Aliquots (approx. 1 nmol of each) of bovine, monkey and human IRBPs were digested with trypsin for 24 h at 37 °C. Digests were chromatographed on a Zorbax C_8 column, elution being with a gradient of 0.05% TFA in water to 0.05% TFA in aq. 70% acetonitrile with detection at 210 nm. (*a*) Bovine IRBP; (*b*) monkey IRBP; and (*c*) human IRBP.

Effect of retinol on the tryptic digestion of IRBP

Prior incubation of portions of bovine IRBP with concentrations of all-*trans*-retinol ranging from 10^{-6} to 10^{-9} M had no effect on the subsequent tryptic-digestion patterns. The patterns for each concentration of retinol showed no marked difference from each other or from the ethanol control (results not shown).

Reversed-phase h.p.l.c. mapping of human, monkey and bovine IRBP tryptic digests

Complete tryptic digests of human, monkey and bovine IRBPs were analysed by C_8 reversed-phase h.p.l.c. (Figs. 3 and 4). A common feature was the predominance of slow-to-be-eluted peptides, with most peptides being eluted between 27 and 44% acetonitrile. These are larger or more hydrophobic peptides than those generally expected for total mapping of soluble proteins and may be a reflection of the large size and hydrophobic nature of the intact protein. With detection at 210 nm (Fig. 3), few similarities in fine detail were seen among the IRBPs of the three species under study, and most of the peptide peaks are seen to differ both qualitatively (retention time) and quantitatively (absorbance). Detection at 280 nm (Fig. 4), which simplifies the peptide map, allowing easier comparison, also showed few similarities.

In addition, tryptic digests of the three IRBPs were analysed by C_{18} reversed-phase h.p.l.c., elution being with 0.05% TFA in a gradient of propan-2-ol (results not shown); this yielded conclusions similar to those reached from the C_8 reversed-phase experiments.

N-Terminal sequences

The *N*-terminal sequences of monkey and bovine IRBPs have been extended to 32 and 34 residues respectively (Tables 1 and 2; Fig. 5). The first 15 residues of both the monkey and bovine *N*-terminal sequences have been previously published by us (Redmond *et al.*, 1985). Taking into account the offset of five residues between the two sequences (Redmond *et al.*, 1985), there

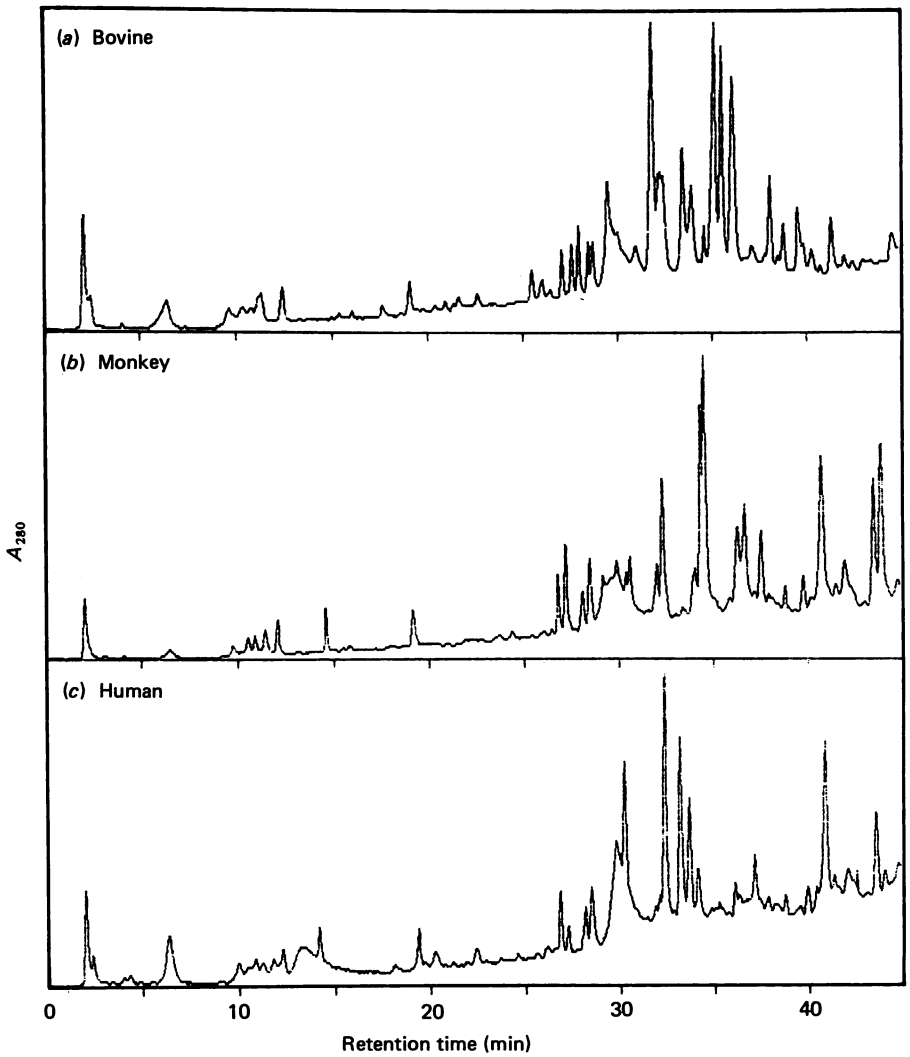

Fig. 4. Comparison of reversed-phase h.p.l.c. tryptic peptide maps of bovine, monkey and human IRBPs

Conditions were as in Fig. 3, except that detection was at 280 nm.

is an overlapping sequence of 27 residues. Except for three unidentified residues in the monkey sequence, there are only two differences between the two sequences. The first is a conservative substitution of an aspartic acid residue at position 13 of the monkey sequence for a glutamic acid residue at position 8 of the bovine sequence, and a lysine residue at position 16 of the monkey sequence for a glutamine residue at position 11 of the bovine sequence. It should be noted, however, that this new analysis identified residue 4 of the monkey IRBP as histidine rather than alanine as previously published (Redmond *et al.*, 1985). Sequencing of the human protein revealed two *N*-termini present in approximately equal amounts (Table 3). We have designated the sequence beginning with Phe as sequence '*n*', whereas the sequence with the five-residue *N*-terminal addition and beginning with glycine we have termed '*n*+5'. A similar, but less complex, situation was observed in the sequencing of the monkey IRBP in which the '*n*' sequence was present to a much lesser extent than was the '*n*+5' sequence (Table 2). The human protein used for sequencing was purified to homogeneity by using the same procedures and criteria for the other IRBPs (Fig. 6). By analysis of the h.p.l.c. chromatograms for each cycle of degradation, together with the knowledge of the major monkey *N*-terminal sequence, we deduced that the two sequences were identical except for an offset of five amino acid residues and that both were completely identical with the *N*-terminal sequences of monkey IRBP (Fig. 5).

DISCUSSION

We have previously shown that there is significant homology between the 15 *N*-terminal residues of monkey and bovine IRBP (Redmond *et al.*, 1985). Bridges *et al.* (1984) and Saari *et al.* (1985) have published the first 14 and 24 residues respectively, and both sequences are essentially identical with ours. We now report that the homology has been extended at least to residues 32 of the monkey IRBP. The human IRBP has been found to display a complex *N*-terminal sequence which has been resolved into two approximately equimolar sequences, '*n*' and '*n*+5', identical except for an offset of five residues; this is also seen to a lesser extent in monkey IRBP. The human and monkey IRBP *N*-termini show

Table 1. *N*-Terminal sequence analysis of monkey IRBP

Cycle no.	Analysis			
	Major sequence ($n+5$)		Minor sequence (n)	
	Residue	pmol	Residue	pmol
1	Gly	598	Phe	153
2	Pro	301	Gln	161
3	Thr	61	(Pro)*	–
4	His	140	Ser	6
5	Leu	424	(Leu)*	–
6	Phe	306	Val	85
7	Gln	317	Leu	131
8	Pro	201	Asp	71
9	Ser	6	Met	70
10	Leu	248	Ala	119
11	Val	102	Lys	50
12	Leu	157		
13	Asp	100		
14	Met	121		
15	Ala	188		
16	Lys	115		
17	Val	128		
18	Leu	104		
19	Leu	–		
20	Asp	48		
21	Asn	94		
22	Tyr	64		
23	–†	–		
24	Phe	71		
25	Pro	94		
26	–†	–		
27	Asn	45		
28	Leu	100		
29	–†	–		
30	Gly	60		
31	Met	29		
32	Gln	26		

* Amino acid residues in parentheses tentatively assigned.
† Residues not identifiable.

Table 2. *N*-Terminal sequence analysis of bovine IRBP

Cycle no.	Analysis	
	Residue	pmol
1	Phe	272
2	Gln	225
3	Pro	178
4	Ser	14
5	Leu	162
6	Val	160
7	Leu	146
8	Glu	44
9	Met	126
10	Ala	148
11	Gln	125
12	Val	121
13	Leu*	–
14	Leu*	–
15	Asp	25
16	Asn	68
17	Tyr	60
18	(Glu)†	11
19	Phe	61
20	Pro	66
21	Glu	14
22	Asn	37
23	Leu	81
24	Met	59
25	Gly	45
26	Met	50
27	Gln	50
28	(Gly)†	9
29	Ala	88
30	Ile	45
31	(Glu)†	17
32	Gln	43
33	Ala	26
34	Ile	32

* Yield of leucine at cycle 13 and 14 not accurately determined.
† Residues in parentheses tentatively assigned.

total identity and share the same differences with the bovine IRBP, which manifests only the '*n*' sequence. Neither the cause of, nor the reason for, this variable processing is clear. It may be the result of 'artefactual' cleavage by proteinases during isolation, although no evidence of further degradation was found. Alternatively, and more interestingly, the observed *N*-terminal may be a result of variable post-translational processing. Variable *N*-terminal sequences have previously been found in rabbit liver microsomal *P*-450 isoenzyme 4 (Fujita *et al.*, 1984) and in porcine adrenocortical cytochrome *P*-450 21-hydroxylase (Yuan *et al.*, 1983). In addition, several imported bovine mitochondrial ATP synthase subunits have been shown to have variable *N*-termini (Runswick *et al.*, 1986; Walker *et al.*, 1985).

Limited tryptic and *S.-aureus*-V8-proteinase digests of the three IRBPs indicate that human and monkey IRBPs are very closely related to each other. Although the proteolytic patterns as revealed by SDS/polyacrylamide-gel-electrophoresis are almost completely identical for the two primate proteins, there are significant differences between the primate and the bovine proteolytic patterns. For example, the time-course analysis of the tryptic digestion of monkey and bovine IRBPs shows that the monkey IRBP is more rapidly cleaved to small fragments than is the bovine IRBP. It should be stressed, though, that the differences beteen the primate patterns and the bovine pattern may be more related to relative accessibility of various cleavage sites during the period of limited digestion or to a few strategically located substitutions than to large differences in the primary structure away from the *N*-terminus. In all three species, however, the smallest immunoreactive fragment whether generated by trypsin or by V8-proteinase digestion, is similar, with an M_r of about 25000.

Although they share similarity in terms of a preponderance of large or hydrophobic peptides, the reverse-phase h.p.l.c. peptide-mapping patterns of the three IRBPs are quite dissimilar in terms of fine structure. This lack of fine detail is probably a reflection of an unknown number of substitutions in the sequences of the three proteins, which, though they may be minor or conservative, do result in chromatographic changes.

In conclusion, it appears that mammalian IRBPs share many similarities, exhibiting a high degree of conserva-

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                1     5        10        15        20        25        30        35
Monkey          G P T H L F Q P S L V L D M A K V L L D N Y ? F P ? N L ? G M Q
Bovine                F Q P S L V L E M A Q V L L D N Y (E)F P E N L M G M Q(G)A I E Q A I
Human 'n'             F Q P S L V L D M A K V L L D N Y ? F P
Human 'n + 5'   G P T H L F Q P S L V L D M A K V L L D
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Fig. 5. *N*-Terminal sequences of monkey, bovine and human IRBPs

Underlined residues are identical with those in the monkey sequence. Both human 'n' and '$n+5$' sequences are given (see the text). Residues in parentheses are tentative assignments. The one-letter amino acid notation is used.

Table 3. *N*-Terminal sequences of human IRBP

Cycle no.	Analysis			
	Sequence $n+5$		Sequence n	
	Residue	pmol	Residue	pmol
1	Gly	161	Phe	98
2	Pro	66	Gln	50
3	Thr*	–	Pro	92
4	His	29	Ser*	–
5	Leu†	–	Leu†	–
6	Phe	39	Val	56
7	Gln	33	Leu	60
8	Pro	34	Asp	18
9	Ser*	–	Met	53
10	Leu	45	Ala	49
11	Val	28	Lys	41
12	Leu	35	Val	51
13	Asp	12	Leu	76
14	Met	23	Leu	89
15	Ala/Val‡	10/13	Asp	13
16	Lys	26	Asn	32
17	Val	16	Tyr	23
18	Leu	3	–§	–
19	Leu	38	Phe	43
20	Asp	10	Pro	22

* Not quantified owing to multiple derivatives.
† Leucine was identified as the residue for both sequences in this cycle; total yield was 108 pmol.
‡ Both alanine and valine were possible assignments; alanine is more likely, however, to be the correct residue.
§ Not identifiable.

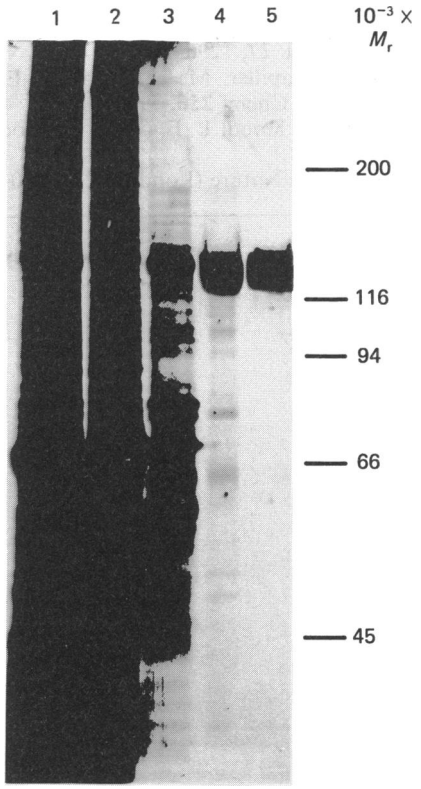

Fig. 6. Purification of human IRBP

Samples from different stages of the purification of human IRBP were electrophoresed on an SDS/8%-polyacrylamide-gel system. The gel was silver-stained. 1, Crude human retina wash; 2, material not binding to concanavalin A–Sepharose; 3, bound material to concanavalin A–Sepharose eluted with α-methyl D-mannoside; 4, peak from anion-exchange chromatography and 5, pool of final purified IRBP preparation from size-exclusion h.p.l.c.

tion, particularly between the two primate proteins. Within this framework, however, significant species-specific differences in fine structure are present, as indicated by the reversed-phase peptide mapping. Further analysis of the proteolytic fragments of IRBP will be of importance in our understanding of the relation of structure to function, possibly identifying binding domains that are important immunologically, in fatty acid and retinoid binding, and in interactions with surrounding plasma membranes.

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