Linkage of Chromosomal Markers on 4q With a Putative Gene Determining Maximal Insulin Action in Pima Indians

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Insulin action in vivo varies widely in nondiabetic Pima Indians. Not all of this variance is attributable to individual differences in obesity, physical fitness, sex, or age, and after correcting for these co-variates, measures of insulin action aggregate in families. Insulin action at maximally stimulating insulin concentrations has a trimodal frequency distribution, particularly among obese individuals. This is consistent with the hypothesis that a codominantly inherited autosomal gene, unrelated to obesity, determines MaxM in the population. Preliminary sib-pair linkage analyses indicated the possibility of linkage between MaxM and the GYPA/B locus (encoding the MNSs red cell surface antigens) on chromosome 4q. To confirm and extend these findings, 10 additional loci on 4q were typed in 123 siblings and many of their parents from 46 nuclear families. The results indicate significant ($P < 0.001$) linkage of the FABP2 and ANX5 loci on 4q with MaxM, and of FABP2 with fasting insulin concentration. No linkage was found between the 4q markers and obesity. Our findings indicate that a gene on 4q, near the FABP2 and ANX5 loci, contributes to in vivo insulin action in Pima Indians. *Diabetes* 42:514–19, 1993

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MaxM, maximal insulin-stimulated glucose uptake; NIDDM, non-insulin-dependent diabetes mellitus; OGTT, oral glucose tolerance test; RIA, radioimmunoassay; RFLP, restriction fragment length polymorphism; EMBS, estimated metabolic body size; PCR, polymerase chain reaction; kb, kilobase; PGIBD, proportion of genes identical by descent.

The Pima Indians of Arizona have the highest reported prevalence of NIDDM of any population in the world (1,2), and among those who develop the disease at a young age, the disease shows strong familial aggregation (3). Insulin resistance is a major risk factor for the disease (3–5), and insulin action in vivo, as measured with the hyperinsulinemic, euglycemic clamp, aggregates in families (6). At maximally stimulating insulin concentrations, insulin action is trimodally distributed (7). These data are consistent with the hypothesis that maximal insulin action is determined by a codominantly inherited autosomal gene in Pima Indians. Consequently, we have sought genetic markers of insulin resistance in this population.

Many Pimas have been typed for several different red cell antigens. Because the chromosomal locations of the genes that determine many of these antigens are known, we began the search for genetic markers of insulin resistance by performing sib-pair linkage analyses between various red cell antigens and insulin action at maximally stimulating insulin concentrations. Preliminary sib-pair analyses indicated possible linkage between maximal insulin action and the glycophorin A/B locus (GYPA/B, determining the MNSs red cell surface antigens) on 4q, the long arm of chromosome 4 (8). To confirm and extend these preliminary results, we performed sib-pair linkage analyses between maximal insulin action and several additional markers on 4q in a larger group of siblings. Linkage analyses with these markers also were performed with percentage of body fat and fasting plasma insulin concentrations. The results indicate linkage between a putative gene determining maximal insulin action in vivo in Pima Indians and genetic markers in the region of 4q26.

TABLE 1
Clinical characteristics of siblings

n	123
Sex (M/F)	56/67
Age (yr)	28 ± 6
Height (cm)	165 ± 8
Weight (kg)	105.3 ± 28.3
Body fat (%)	37 ± 7
2-h postload glucose (mM)*	7.9 ± 1.8
Hyperinsulinemic, euglycemic clamp	
Plasma glucose (mM)	5.3 ± 0.4
Log_{10} plasma insulin (pM)	4.25 ± 1.0
Glucose uptake rate ($\mu M\ min^{-1} \cdot kg^{-1} \cdot EMBS^{-1}$)†	43.3 ± 11.6

Data are means ± SD.
*Plasma glucose concentration 2 h after ingesting 75 g glucose.
†EMBS = fat-free body mass + 17.7 (see METHODS).

RESEARCH DESIGN AND METHODS

The subjects are members of the Gila River Indian Community who have participated in a longitudinal study of the development of NIDDM since 1965 (1–3). Information on parents, siblings, and children of each individual has been collected and updated since the inception of the study. Approximately every 2 yr, beginning at 5 yr of age, individuals are asked to come for examination after an overnight fast. Typing for several red cell antigens, including the MNSs antigens, has been performed on each subject presenting for the examination. Individual genomic DNA for typing of additional markers (see below) was isolated from Epstein-Barr virus–transformed lymphocyte cultures, or directly from peripheral white blood cells obtained from participants in this study (9).

A subset of individuals from this population was admitted to the clinical research ward and fed a weight-maintaining diet. After 3 days, each person underwent several tests on different days including a 75-g OGTT (10), an underwater weighing to determine body composition including the fat-free body mass (11), and a two-step hyperinsulinemic, euglycemic clamp to measure insulin action in vivo as described in detail elsewhere (6,7,11). Briefly, a low-dose, primed-continuous insulin infusion (290 $pmol \cdot m^{-2} \cdot min^{-1}$) was started at ~0800 and continued for 100 min. Simultaneously, a variable 20% glucose infusion was given to maintain the plasma glucose concentration at the basal level, and plasma glucose concentrations were determined every 5 min throughout the test. Immediately after the low dose insulin infusion, a high-dose, primed-continuous infusion (2900 $pmol \cdot m^{-2} \cdot min^{-1}$) of insulin was given for another 100 min. This higher insulin infusion rate induces close to maximal insulin stimulation of glucose uptake. The mean plasma glucose and insulin concentrations achieved during the high-dose insulin infusion are given in Table 1.

To account for differences in metabolic body size among subjects, glucose uptake rates were normalized for the EMBS (EMBS = fat-free body mass + 17.7). This estimate of metabolic body size was derived from resting metabolic rate measurements with indirect calorimetry as described elsewhere (12). Glucose uptake rates also were adjusted for steady-state plasma glucose concentrations during the clamp, as suggested by Best et al. (13).

Plasma glucose concentration was measured by the glucose oxidase method with a Beckman glucose analyzer (Fullerton, CA). Plasma insulin concentrations were determined either by the Herbert modification (14) of the RIA of Yalow and Berson (15) or, more recently, with a radioassay analyzer (Concept 4, ICN, Harshaw, PA). An adjustment was made to the insulin assay results obtained with the radioassay analyzer to make the results comparable with that obtained by the earlier RIA. The adjustment equation was calculated from assays performed with both methods on several hundred samples representing a wide range of plasma insulin concentrations. The fasting insulin levels used in these analyses are the mean of two measurements made on each of three different days.

DNA marker typings. Ten informative DNA markers on 4q were selected to extend the initial finding with the

TABLE 2
Allele frequencies of chromosomal 4q markers

		Allele number							
Marker	*n**	1	2	3	4	5	6	7	8
D4S231	77	0.188	0.143	0.150	0.468	0.032	0.006	0.013	
D4S193	80	0.125	0.444	0.362	0.069				
D4S101	70	0.029	0.093	0.228	0.650				
D4S191	79	0.360	0.019	0.618	0.003†				
FABP2	207	0.278	0.041	0.669	0.007	0.005			
ANX5	85	0.624	0.376						
D4S194	81	0.030	0.093	0.259	0.599	0.019			
D4S175	156	0.054	0.002	0.022	0.109	0.240	0.029	0.003†	0.541
D4S192	80	0.200	0.113	0.300	0.069	0.312	0.006		
GYPA/B	—‡	0.260§	0.450§	0.090§	0.200§				
D4S171	82	0.079	0.402	0.250	0.062	0.207			

*No 2 subjects were first-degree relatives.
†In the siblings, an additional allele was found that was not present in the unrelated individuals. The frequency of this new allele was estimated by considering it to occur only once in twice the number of unrelated individuals.
‡Previously determined by using maximum likelihood methods in a large population survey (19).
§MNSs genotypes: 1 = MS, 2 = Ms, 3 = NS, 4 = Ns.

TABLE 3
Primer sequences of PCR-based 4q markers used in the linkage study

Marker	Primer name	Sequence (5′→3′)
D4S231	YUNCA9-10FWD	AGATGAGTATGTTATTATACC
	YUNCA9-10REV	TGCTAGAGTTCCCTAGTG
D4S193	Mfd142CA	ACAACCCCATTTGTGAAGAC
	Mfd142GT	TTTATAGAAAATTTAGCATGGA
D4S191	Mfd138CA	AATAGGGAGCAATAAGGTGT
	Mfd138GT	TTTTTATTATGTTTGCTGCTC
FABP2	FABP2.PCR2.1	GTAGTATCAGTTTCATAGGGTCACC
	FABP2.PCR2.2	CAGTTCGTTTCCATTGTCTGTCCG
ANX5	ANX5.PCR1.1	TTGAATTCGCCTACCTTGCAGAGACCCT
	ANX5.PCR1.2	CTAAGCTTAGTCATCTTCTCCACAGAGC
D4S194	Mfd146CA	CTAAGGGATGACTATATCCT
	Mfd146GT	GATCTGATTACATGTCCGT
D4S175	Mfd38CA	ATCTCTGTTCCCTCCCTGTT
	Mfd38GT	CTTATTGGCCTTGAAGGTAG
D4S192	Mfd140CA	TTCAAGCACTGAAAGGGATG
	Mfd140GT	GATCCTCAAGTGGAGTTTG
D4S171	Mfd22CA	TGGGTAAAGAGTGAGGCTG
	Mfd22GT	GGTCCAGTAAGAGGACAGT

Information about all listed primer sequences also is available from the Genome Database (Johns Hopkins University, Baltimore, MD).

MNSs blood group marker (Table 2). For D4S101, an RFLP was typed by Southern blot hybridization as described (16). Polymorphisms at markers ANX5, D4S171, D4S175, D4S191, D4S192, D4S193, D4S194, D4S231, and FABP2 were typed by the PCR. A diallelic polymorphism at ANX5 was determined by *Pvu* II digestion of a 1.5-kb PCR product amplified as described previously (17). Allelic variants at the remaining eight markers were based on length differences of short simple DNA repeats (microsatellites), which were PCR amplified with marker-specific primers synthesized on a DNA synthesizer (Model 391, Applied Biosystems, Foster City, CA). Sequence information for the microsatellite markers was provided by Dr. J. Murray (University of Iowa) and is given in Table 3. This information also is available through the Genome Database at the Johns Hopkins University (Baltimore, MD).

Individual DNA samples (50 ng) were amplified in a total volume of 10–12.5 μl for 25–30 cycles using a DNA Thermal cycler 480 or GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of a denaturing step at 94°C for 30–60 s, primer annealing for 1 min at a temperature appropriate for the primer set of each individual marker, and extension at 72°C for 30–60 s. An aliquot of the PCR product (2–4 μl) was mixed with an equal volume of 1× PCR buffer containing 0.1–0.2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) plus one of the marker-specific primers end-labeled with γ-^{32}P-ATP (6000 Ci/mmol, DuPont-NEN, Boston, MA). This mixture was subjected to one additional amplification cycle, the labeled products were denatured, and separated on a sequencing gel. Autoradiography was performed without an intensifying screen for 16–18 h at room temperature or at −70°C. The results were scored independently by two investigators. Because no size comparison was made with allele sizes published for individual markers, the numeric allele designations used in this study do not necessarily match published allele designations. Families were excluded from the analysis if any siblings were incompatible with other members of the family at any locus (indicating nonpaternity).

Linkage analyses. Linkage analyses were performed on sib-pairs with the Statistical Analysis for Genetic Epidemiology package (S.A.G.E., see ACKNOWLEDGMENTS). With this method, linkage is assessed for dichotomous or continuous traits from the correlation between the squared sibling difference in a trait, and the PGIBD. A one-tailed Student's *t* statistic is used to test the significance of the correlation. Only $P < 0.001$ was considered significant as suggested by Ott (18). If any sib-pair squared difference is >3SD from the mean sib-pair squared difference at a particular PGIBD, then the correlation is determined after deleting these outlying values. The linkage results reported herein are after deleting these outlying values. In no case, however, were more than two sib-pairs deleted. Linkage analyses were performed on percentage of body fat, the $\log_{10}$ of the fasting plasma insulin concentrations, and maximal insulin action in vivo. Maximal insulin action in vivo is distributed as a mixture of three normal distributions in this population (7) and is not skewed toward higher or lower values, therefore, the MaxM values were not log-transformed before analysis.

RESULTS

Siblings. Only subjects without NIDDM (10) were included in the analysis to avoid the potentially confounding effects of secondary changes in fasting insulin concentrations and/or insulin action in vivo attributable to the diabetic state itself. However, if data were available from an individual before NIDDM developed, these data were included. In addition, only data from individuals with a body fat >25% were included in the analysis because the trimodal frequency distribution of maximal insulin

TABLE 4
Sib-pair linkage analyses between 4q markers and percentage of body fat and $\log_{10}$ fasting insulin concentrations and MaxM adjusted for sex and percentage of body fat

Marker	Mean PGIBD*	Sib-pairs (*n*)	*P* values for linkage: Body fat (%)	*P* values for linkage: Adjusted $\log_{10}$ fasting insulin	*P* values for linkage: Adjusted MaxM
D4S231	0.480	109	0.214	0.070	0.353
D4S193	0.499	116	0.205	0.733	0.726
D4S101	0.493	101	0.162	0.087	0.051
D4S191	0.500	116	0.143	0.013	0.076
FABP2	0.479	123	0.606	0.0004	0.0008
ANX5	0.528	123	0.068	0.015	0.0005
D4S194	0.471	115	0.278	0.236	0.055
D4S175	0.474	122	0.113	0.017	0.083
D4S192	0.475	108	0.183	0.235	0.501
GYPA/B	0.496	128	0.121	0.175	0.882
D4S171	0.532	120	0.801	0.274	0.552

*For each marker, this value was not statistically different from 0.50, which is consistent with a Mendelian segregation of alleles at each marker in this population and lack of nonpaternity.

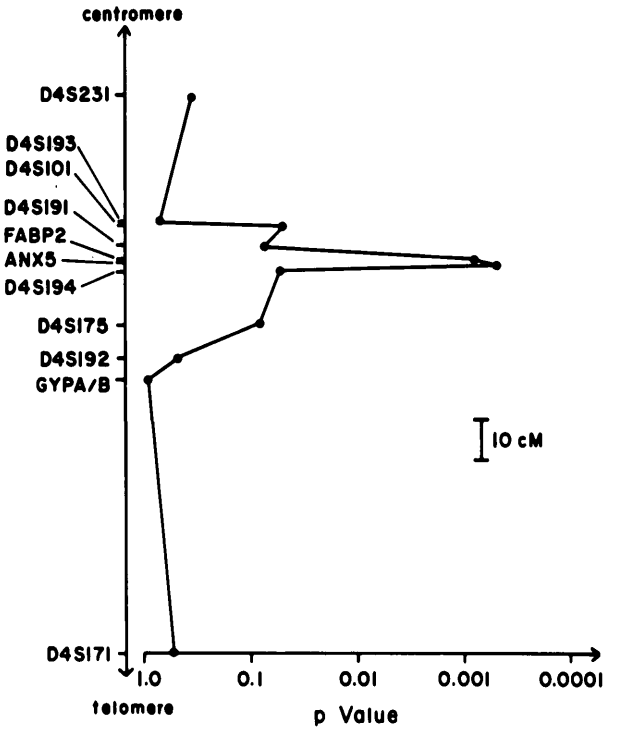

FIG. 1. Plot of *P* values from sib-pair linkage analyses between MaxM and 11 markers on 4q.

action was clearest in obese individuals alone (7). Measurements of insulin action in vivo, fasting insulin concentrations, and body composition were available on 123 siblings from 46 nuclear families with an average of 2.7 siblings per family. The number is sib-pairs (*P*) in a family of *N* siblings is given by the formula: $P = N(N - 1)/2$. The number of sib-pairs from a group of 123 siblings will therefore depend on the size of the families studied. In this study, we had a maximum of 123 sib-pairs for analysis from the 123 siblings studied. The characteristics of the siblings are given in Table 1.

Sib-pair linkage analyses. The allele frequencies of each of the markers were estimated on a set of members of the Gila River Indian Community, no two of whom were first-degree relatives (Table 2). The allele frequencies at the GYPA/B locus (MNSs) were determined previously in a large population survey (19).

GYPA/B (MNSs) typing was available on 128 sib-pairs and all but 6 parents from the 46 nuclear families. Most of the siblings were typed at the 10 additional loci on 4q (Table 2) and, in most cases, at least one parent was also typed.

Sib-pair linkage analyses were performed between the markers and percentage of body fat, $\log_{10}$ fasting insulin concentrations, and MaxM rates. These values were adjusted for sex differences by separately standardizing the values in men and women to a mean of zero and 1SD. The $\log_{10}$ fasting insulin concentrations and MaxM also were adjusted for percentage of body fat by using linear regression analysis.

The results of the sib-pair linkage analyses given in Table 4 indicated a significant linkage of FABP2 and ANX5 with MaxM adjusted for sex and percentage of body fat (Fig. 1) and a linkage of FABP2 with $\log_{10}$ fasting insulin level. Percentage of body fat was not linked to any of the markers analyzed.

Linkage disequilibrium. Because of the significant linkage results between the FABP2 and ANX5 loci and a putative locus determining MaxM (adjusted for percentage of body fat and sex), an analysis of linkage disequilibrium between these two markers, and adjusted $\log_{10}$ fasting insulin concentrations, and MaxM also was undertaken. Only results from individuals who were full-blooded Pima/Pagago Indians were analyzed to avoid the potential confounding effects of racial admixture (20). Also, additional subjects were included in this analysis on whom phenotype and genotype data were available but who were not siblings.

For the ANX5 locus, the analysis was performed by comparing the mean $\log_{10}$ fasting insulin concentration and mean MaxM of individuals who were homozygous for either allele 1 ($n = 65$) or allele 2 ($n = 19$), nonpaired Student's *t* tests were used. No significant associations were found.

For the FAB2 locus, the analysis was performed by comparing the mean adjusted $\log_{10}$ fasting insulin concentration and mean adjusted MaxM of individuals who were homozygous for each of the alleles if there were ≥10 individuals in a group. This reduced the analysis to performing non-paired Student's *t* tests on the mean values of individuals who were homozygous for allele 1 ($n = 13$) or allele 3 ($n = 70$). The mean ± SD adjusted $\log_{10}$ fasting insulin concentration of those homozygous for allele 1 (−0.54 ± 0.23) was lower than the mean ± SD of those homozygous for allele 3 (0.18 ± 0.09; $P = 0.007$). Consistent with this difference in fasting insulin levels, the mean adjusted MaxM was higher (0.25 ± 0.27) in those homozygous for allele 1 than those homozygous for allele 3 (−0.36 ± 0.11; $P = 0.042$).

DISCUSSION

Insulin action in vivo has a large variance in the Pima Indian population (6). This variance is not entirely attributable to individual differences in the degree of obesity, physical fitness, sex, or age, and the variance not attributable to these covariates aggregates in families (6), indicating possible genetic determinants. An additional indication of genetic determinants of insulin action in Pimas is its frequency distribution. Insulin action, particularly at maximally stimulating insulin concentrations, is trimodally distributed (7), and the best separation of the three components is observed among the most obese individuals (7). These data are consistent with the hypothesis that insulin action, particularly at maximally stimulating insulin concentrations, is determined by a codominantly inherited autosomal gene with two alleles; and that this putative gene determines insulin action that is in addition to any effects of genes or environmental factors that result in obesity.

To locate this putative gene, we initially performed sib-pair linkage analyses between maximal insulin action and various red cell antigens. Because the chromosomal location of most of the genes determining these antigens was known, any evidence of genetic linkage might indicate the presence of a gene for insulin resistance near one of these loci. One of the first red cell antigen systems to be analyzed was the MNSs system, determined by the GYPA/B locus in the region of 4q28–31, because typings were available in almost all the Pimas, and because it was quite polymorphic within the population (19). Preliminary sib-pair linkage analyses in a limited number of nuclear families indicated possible linkage between this locus and maximal insulin action (8).

To confirm (or refute) these initial findings, we typed these and additional siblings for more markers on 4q. The results demonstrate linkage between a region of chromosome 4, presumably containing a putative gene determining MaxM, and the FABP2 and ANX5 genes, which themselves are separated by ~1 centiMorgan in the 4q26 region (Fig. 1). Another phenotype that gives an additional, albeit a less precise, estimate of insulin action in vivo, $\log_{10}$ fasting insulin concentration, is also linked to this region. Thus, linkage was observed with more than one phenotypic manifestation of insulin action, and with two closely linked markers. This strengthens the conclusion that a gene near FABP2/ANX5 exists that determines maximal insulin action in vivo in Pima Indians. The FABP2 locus also was found to be in linkage disequilibrium with the putative allele determining insulin resistance in this population. This would suggest a close proximity between those two loci. However, the linkage disequilibrium results need to be interpreted with caution because of the small numbers of individuals in the analysis who were homozygous for allele 1 at the FABP2 locus.

No significant linkage was observed between any marker and insulin-stimulated glucose uptake rates during the low-dose insulin infusion (subMaxM) (data not shown). At these low plasma insulin concentrations (~700–800 pM), most Pimas have little or no increase in glucose uptake above the rate of basal endogenous glucose production (21).

Sib-pair linkage analysis was used in this study rather than the lod-score method primarily because the measurements of insulin action in vivo made on the Pimas in the last decade were performed in one generation. The sib-pair method has several advantages over the lod-score method. The sib-pair method is model independent, and it is not necessary to know, or assume, gene frequencies and penetrances of the genes determining the traits of interest, or in the case of quantitative traits, as in this study, the means and variances of genotypes. A disadvantage of the sib-pair method is that it does not result in an estimate of the recombination fraction between a marker and a trait locus. For example, from the sib-pair analysis, we cannot estimate the genetic distance (in centiMorgans) between the FABP2 and ANX5 loci, and the putative gene for insulin action.

A potential pitfall in sib-pair analyses arises from unrecognized nonpaternity. Offspring of the same mother but with different fathers may be compatible with other offspring at any given locus, but are statistically more likely to be discordant at a marker locus and for any other genetic trait than full siblings. Thus, unrecognized nonpaternity increases the variance in the squared sibling trait differences, and results in greater discordance at the tested marker locus, thereby spuriously simulating linkage. This seems an unlikely explanation for these findings, however, because on average, as shown in Table 4, the proportion of alleles shared by the sib-pairs at each locus was not statistically different from 0.50, as would be expected under the assumption of correct parental designation.

Finally, it is worthwhile considering whether or not FABP2, the intestinal fatty acid–binding protein gene, or ANX5, the annexin V gene, are in fact genes that might affect insulin action in vivo in the Pimas. Although fatty acid metabolism has historically been linked to insulin resistance since Randle's original work (22), we cannot specifically link an abnormality in an intestinal fatty acid–binding protein to insulin action. Annexin V is a member of a family of intracellular calcium-dependent, phospholipid-binding proteins (23) with no clearly established physiological function. Therefore, this protein also cannot be specifically implicated to cause insulin resistance at this time. No other loci are known to be closely linked to FABP2 or ANX5, but efforts are underway to identify candidate genes in this region.

In conclusion, sib-pair analyses in Pima Indians have provided evidence for genetic linkage between the FABP2/ANX5 loci on 4q26 and a putative gene determining insulin action in vivo. This putative gene appears to be distinct from genes or environmental factors that result in obesity and thereby affect insulin action. Whether this putative gene is a minor or major genetic determinant of insulin action in Pimas remains to be established. Also, because insulin resistance is a major risk factor for NIDDM in the Pimas, it will be important to determine whether NIDDM also is linked to the putative locus identified on 4q. These studies are currently in progress.

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