of the β1 domain of IA^d on the N terminus of the peptide or the linker amino acids on the C terminus.

In both cases treatment of the peptide–MHC covalent complex with thrombin to cleave the linker between them only modestly improved T-cell hybridoma recognition (Fig. 4*b*, *d*). It is therefore likely that usually the linker extends from the C-terminal end of the peptides around the side rather than over the top of the class II α-chain α-helix in order to reach the N-terminal end of the class II β-chain (Fig. 1).

These experiments shows that it is possible to produce a covalent complex of peptide and class II protein which can be recognized by most T cells specific for the combination. Constructions of this type should be useful in experiments on the structure of T-cell receptor–ligand interactions and in fact, in the case of the IA molecule, may be the only way to generate soluble class II/peptide complexes in reasonable quantities. In preliminary experiments we have found that proteins like this, with their associated transmembrane regions restored, are well expressed on the surface of mouse B cells and fibroblasts where they are recognized by the appropriate T cell and inhibit presentation of other peptides by at least 100-fold (data not shown). The expression by cells of class II proteins bound entirely, or mostly, to a single peptide will be invaluable in studies of positive selection of T cells. They may also allow very efficient induction of tolerance *in vivo* to particular class II/peptide complexes, a phenomenon that may be of therapeutic significance[20]. □

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Genetically based *N*-acetyltransferase metabolic polymorphism and low-level environmental exposure to carcinogens

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THE metabolic activation or inactivation of carcinogens varies considerably in human populations, and is partly genetically determined[1,2]. Inter-individual variability in the susceptibility to carcinogens may be particularly important at low degrees of environmental exposure. Examples of probable human carcinogens that present widespread low-dose exposures are environmental tobacco smoke and diesel exhaust[3,4]. We have determined levels of DNA adducts in bladder cells and of 4-aminobiphenyl–haemo-

TABLE 1 Levels of ABP-haemoglobin adducts by nicotine–cotinine in 24-h urine and acetylator phenotype in 97 volunteers

ABP-haemoglobin adduct concentration (pg per g haemoglobin)

Nicotine + cotinine†	Whole group Median	Whole group Mean	Acetylator phenotype Rapid* (median)	Acetylator phenotype Slow* (median)	Per cent increase in slow
0 (n = 44)	22.5	28.5	13	27	107
< 1.5 (n = 25)	65	79.5	52	99	90
1.5–2.4 (n = 9)	128	123.7	92	132	43
2.5 + (n = 19)	125	135.3	128	119	−7

Number of subjects with ABP adducts above/below median

	Acetylator phenotype Rapid	Acetylator phenotype Slow	OR	95% CI
0 (median 22.5)	1/10	21/12	17.5	2.0–153
> 0 (median 104)	9/14	18/12	2.3	0.8–7.0

Slow acetylators are those with a 5-acetylamino-6-formylamino-3-methyluracil:1-methylxanthine ratio <0.6. The odds ratios (OR) represent the probability of having a high concentration of adducts (greater than the median value) for slow versus rapid acetylators. 95% CI, 95% confidence interval. For the analysis of ABP-haemoglobin adducts, isolated haemoglobin was purified by dialysis and then hydrolysed with base to release the parent amine. After extraction into hexane, the amine was derivatized to form pentafluoropropionamide. Quantification was by comparison of peak areas produced by the analyte and an internal standard (4'-fluoro-4-aminobiphenyl) upon capillary gas chromatography, using negative-ion chemical-ionization mass spectrometry for detection. For determination of the acetylator status, urine samples were collected 5 h after consumption of one standardized cup of coffee. Caffeine and its metabolites were extracted as described[6]. The extracts were analysed by HPLC using a program that separated caffeine and all its known metabolites. Haemoglobin adducts were analysed at MIT, Cambridge; the metabolic phenotype for the NAT polymorphism was determined at NCTR, Jefferson; urinary cotinine and nicotine were measured at IARC, Lyon. * P value for trend <0.0001. † units, μmol per mmol creatinine; n represents number of subjects.

TABLE 2 Correspondence between *NAT2* genotype and acetylator phenotype

Genotype (number of mutations)	*n*	Acetylator phenotype Rapid	Slow	OR	(95% CI)	Correlation coefficients: AFMU/1 − X versus nicotine–cotinine
0	8	6	2	1.0	-	0.03 ($P=0.9$)
1	13	5	8	4.8	(0.7–33)	0.37 ($P=0.2$)
2+	20	1	19	57.0	(7.0–460)	0.10 ($P=0.7$)
				ABP adducts		
	n			Mean	Median	s.e.
0	8			43.2	22	18.5
1	13			68.7	56	17.7
2+	20			69.7	65	12.1

OR, odds ratio; 95% CI, 95% confidence interval; correlation coefficients: AFMU/1 − X ratio versus nicotine–cotinine (*P* values), by genotype; and mean 4-aminobiphenyl–haemoglobin adducts, by genotype (s.e., standard error). The genotype for the *NAT2* gene was determined using PCR and RFLP on DNA extracted from buffy coat. Four mutations have been analysed (M1, M2, M3, M4)[8]. The genotype was determined at the NCI, Bethesda. *n* Represents number of subjects.

globin adducts in 97 volunteers, together with the *N*-acetylation non-inducible phenotype, the corresponding genotype, and the levels of nicotine–cotinine in the urine. We find that among the slow acetylators, 4-aminobiphenyl adducts were higher than in rapid acetylators at low or null nicotine–cotinine levels, whereas the difference between slow and rapid acetylators was less evident at increasing nicotine–cotinine levels. The *N*-acetyltransferase genotype is highly predictive of the acetylation phenotype. Our results indicate that the clearance of low-dose carcinogens is decreased in the genetically based slow-acetylator phenotype. Such genetic modulation of low-dose environmental risks is relevant to 'risk assessment' procedures.

A random sample of smokers (50) and one of non-smokers (50) were enrolled from a population of healthy male blood donors (ages 45–64). After obtaining informed consent, we collected detailed questionnaire data, blood (20 ml) and 24-h urine. After consumption of coffee (1 standarized cup), urine was again collected (5 h). Exfoliated bladder cells were successfully obtained from the urine of 73 subjects, and DNA adducts were examined. The metabolic phenotype for the *N*-acetyltransferase (NAT) polymorphism was determined by measurement of caffeine metabolites in 5-h urine. A ratio of 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine of 0.6 was used to separate slow from rapid acetylators[5,6]. The *N*-acetyltransferase *NAT2* genotype was determined using two different polymerase chain reaction (PCR) methods on DNA extracted from buffy coat[7,8]. Cotinine and nicotine were measured in the urine as markers of recent exposure to tobacco smoke. All phases of the study, including laboratory analyses, were blind. Methods[5,9] and some results have been described elsewhere[10,11].

Table 1 shows the mean and median concentrations of 4-aminobiphenyl (ABP)-haemoglobin adducts by levels of urinary cotinine/nicotine, and by acetylator phenotype. Among non-smokers, 44 had no cotinine or nicotine in the urine, but only 3 had no detectable ABP-haemoglobin adducts in the blood. This observation is consistent with the very low half-life of cotinine and nicotine.

There is a convex dose–response relationship between ABP-haemoglobin adduct levels and increasing cotinine–nicotine levels, a relationship that was previously observed[9] and parallels a similar relationship for smoking and bladder cancer[5]. Analysis by acetylator status reveals a different distribution. At low cotinine–nicotine levels, slow acetylators include a higher proportion of subjects with high adduct levels. Increasing cotinine–nicotine levels result in a decreasing proportion of slow acetylators with high adduct levels (Table 1). The odds ratio of slow versus rapid acetylators having a concentration of adducts greater than the median is 17.5 among the subjects with no detectable nicotine or cotinine in the urine (95% confidence interval, 2.0–153), and 2.3 (0.8–7.0) among those with nicotine–cotinine greater than zero. Those who had nicotine–cotinine levels equal to zero included 32 subjects not exposed to environmental tobacco smoke in the 24 h preceding urine collection. Among these, there were 0/9 rapid acetylators with ABP adduct levels higher than the median, whereas the slow acetylators included 9 subjects above the median and 14 below, respectively (odds ratio, infinity). In a multivariate model including four levels of nicotine–cotinine and the acetylator status as independent variables, the difference between slow and rapid acetylators was statistically significant ($T=3.2$; $P=0.0018$).

Table 2 shows the results of *NAT2* genotype analysis. DNA was successfully extracted from 82 subjects. In 15, DNA amplification failed because of technical problems. In the remaining 67, a method based on oligo-specific amplification was used[7]. In 41 of these, a second technique based on restriction-fragment length polymorphism (RFLP) was used[8]. Because the latter method was more sensitive and predictive of the phenotype both in this and a previous study[8], only data for these 41 samples are shown (23 non-smokers and 18 smokers). Four mutations of the gene *NAT2* have been analysed. Only one subject had three mutations, and he had the slow phenotype. The subjects who had at least two mutations had a probability of being slow acetylators 57 times higher than those with no mutation. The correlation coefficients between cotinine–nicotine levels and the 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine ratio were not statistically significant within each genotype, suggesting that there was no induction of the enzyme by smoking. The level of ABP-haemoglobin adducts increased with increasing *NAT2* mutations.

TABLE 3 Presence/absence of DNA adducts 2 and 4 in exfoliated bladder cells, by acetylator phenotype in 39 subjects

Presence or absence	Acetylator phenotype Rapid	Slow
Adduct 2		
No	12	17
Yes	3	7
OR (95% CI)	1.6 (0.3–7.8)	
Adduct 4		
No	8	8
Yes	7	16
OR (95% CI)	2.3 (0.6–8.6)	

Adduct 4 is qualitatively similar to *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl. 24-h urine was centrifuged to collect exfoliated urothelial cells on filters and DNA was isolated from cells. ^{32}P-postlabelling was done under conditions of ATP excess. After autoradiography, individual adducts were visualized, excised and counted. Carcinogen–DNA adduct levels were calculated from the relative adduct labelling:

$$\frac{\text{c.p.m. adducts}}{\text{c.p.m. unadducted nucleotides}} \times 10^9$$

Duplicates (at least) of each sample were analysed separately. DNA adducts were determined at the University of Cincinnati.

Table 3 presents information on DNA adducts in exfoliated bladder cells. Sufficient DNA to detect one carcinogen–DNA adduct per 10^9 normal nucleotides was obtained for 39 subjects (21 non-smokers, 18 smokers). Overall, 12 adducts were found. Two of these (adducts 2 and 4) were moderately associated with smoking habits. Adduct 4 had a correlation coefficient with ABP-haemoglobin adducts of 0.6 ($P=0.01$). This adduct was qualitatively similar to the adduct formed by *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl in the bladders of dogs treated with ABP, and also found in biopsies of human subjects with bladder cancer[11–13]. An association between the presence of adduct 2 or 4 in the exfoliated bladder cells and the slow acetylator phenotype is evident, although not statistically significant.

It is likely that under exceptional exposure conditions (very high levels of exposure) the individual susceptibility is irrelevant. In fact, in a plant where workers were engaged in 2-naphthylamine manufacturing, 15/15 (100%) developed bladder cancer[14]. However, low-dose exposure to carcinogens is widespread and its effects are likely to be modulated by genetic susceptibility. For example, environmental tobacco smoke and possibly vehicle exhaust are sources of ABP in non-smokers. Diesel exhaust contains 4-nitrobiphenyl[4], which is converted to ABP in the body[15]. If the population shows genetic heterogeneity in the susceptibility to chemicals, societal and regulatory decisions must be made that focus the risk assessment process on the most susceptible. □

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The stress-activated protein kinase subfamily of c-Jun kinases

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THE mitogen-activated protein (MAP) kinases Erk-1 and Erk-2 are proline-directed kinases that are themselves activated through concomitant phosphorylation of tyrosine and threonine residues[1–4]. The kinase p54 (M_r 54,000), which was first isolated from cycloheximide-treated rats, is proline-directed like Erks-1/2, and requires both Tyr and Ser/Thr phosphorylation[3,5,6] for activity. p54 is, however, distinct from Erks-1/2 in its substrate specificity, being unable to phosphorylate pp90^{rsk} but more active in phosphorylating the c-Jun transactivation domain[5,7,8]. Molecular cloning of p54 reveals a unique subfamily of extracellularly regulated kinases. Although they are 40–45% identical in sequence to Erks-1/2, unlike Erks-1/2 the p54s are only poorly activated in most cells by mitogens or phorbol esters. However, p54s are the principal c-Jun N-terminal kinases activated by cellular stress and tumour necrosis factor (TNF)-α, hence they are designated stress-activated protein kinases, or SAPKs. SAPKs are also activated by sphingomyelinase, which elicits a subset of cellular responses to TNF-α (ref. 9). SAPKs therefore define a new TNF-α and stress-activated signalling pathway, possibly initiated by sphingomyelin-based second messengers, which regulates the activity of c-Jun.

Amino-acid sequences derived from tryptic peptides of purified p54 align with the consensus Ser/Thr kinase catalytic domain[10]. Based on these sequences, a 467-base-pair (bp) rat brain complementary DNA probe was generated by polymerase chain reaction (PCR) and used to screen a rat brain cDNA library, yielding four classes of p54 cDNAs (Fig. 1*a*): αI encoded a protein containing all the tryptic peptides derived from rat liver p54; β and a partial γ-sequence encoded polypeptides closely related to αI (88–90% identity, respectively; Fig. 1*a*); αII, which probably arises from alternative splicing of the αI transcript, is identical to αI except for a 71-bp region encoding a substitution of 15 amino acids in subdomains IX and X (Fig. 1*a* and ref. 10). The relative molecular masses of the predicted proteins encoded by the full-length clones are: αI, 48,076; αII, 47,986; and β, 48,095. Northern blots of messenger RNA from several tissues revealed ubiquitous but low expression of all three genes (data not shown).

Alignment of the p54 catalytic domains with those of mammalian and yeast MAP kinases (*KSS1*, *HOG-1*, *FUS3*, *SLT-2*, *spk-1* and *erk-1*; refs 11–14) shows that the degree of homology shared by the p54s with mammalian *erk-1* (43–44%) and with the kinases from lower eukaryotes (41–44%) is almost the same. However, the sequence of *erk-1* is closer to these yeast kinases (49–56% identity) than it is to the p54s. Thus the yeast MAP kinases are unlikely to be functional homologues of the p54s.

p54 isoforms contain the amino-acid sequence TPY at position 183–185, in a domain analogous to the TEY sequence of Erks-1/2 (ref. 15). Erks-1/2 are activated through dual phosphorylation by MAPK or Erk kinases (MEKs)[16–18]. But as neither dephosphorylated liver p54 nor bacterially expressed p54 is (re)activated *in vitro* by Erk-1/2-specific MEKs under conditions favouring complete activation of Erks-1/2, the p54s are probably regulated by (a) distinct upstream activator(s).

A polyclonal antiserum raised against the p54 β-isoform immunoprecipitates *in vitro*-translated p54α and β, but not Erk-1 (Fig. 1*b*). It also precipitates a cycloheximide-activated GST–c-Jun kinase from NIH3T3 cells (Table 1). We used this p54-specific immune complex assay of GST–c-Jun phosphorylation to investigate the regulation of p54 kinase activity in a variety of cell lines (Table 1*a*). For comparison, Erks-1/2 activity in the same extracts was assayed, after Mono-Q chromatography, using myelin basic protein (MBP) as a substrate.

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