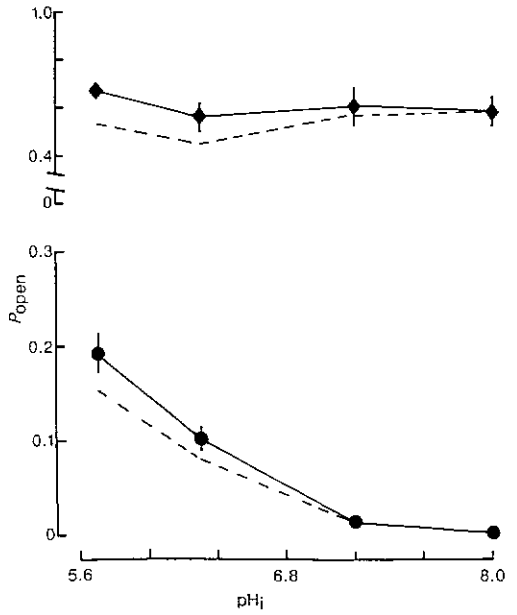

FIG. 3 The effect of pH_i on P_{open} in the absence (◆, upper graph) and presence (●, bottom) of 1 mM $MgCl_2$ and 0.5 mM ATP. Note the difference in ordinate scales. The data are pooled from 16 patches and points show the mean ± s.e.m., unless obscured by the symbol. In some patches there were too many channels to measure P_{open}, so values in the presence of Mg^{2+} and ATP (●) were calculated by multiplying the relative current in Mg^{2+} and ATP, normalized to the control current in the same patch, by the mean P_{open} (◆) measured from patches where this could be resolved under control conditions. The dashed lines show the effective 'P_{open}' after accounting for the reduction in unitary amplitude, relative to that at pH_i 8.0, caused by a decrease in pH_i.

experiments). This change in blocking efficiency was also observed when the ATP was accompanied by excess Mg^{2+}; under these conditions the predominant form of ATP is Mg-ATP^{2-}. The concentration of this complex is 0.36 mM at pH 7.2, and is reduced by only 14% to 0.31 mM at pH 6.3. In contrast to the block of these channels in pancreatic β-cells, there was relatively little difference between the block by 0.5 mM ATP in the absence and presence of Mg^{2+}, indicating that Mg-ATP^{2-} is also effective at inhibiting K_{ATP}-channel activity in skeletal muscle. Figure 2 shows the results of an experiment with a patch held at −3 mV where the action of Mg^{2+} and ATP is compared with control conditions at pH_i 7.2 and 6.3. It is evident that the block by Mg^{2+} and ATP is much less at pH_i 6.3 than at 7.2. In five experiments the mean fractional activity remaining at pH_i 7.2 was 0.02 ± 0.01, compared with 0.18 ± 0.02 at pH_i 6.3. In contrast to the inhibitory effect of Mg^{2+} on K_{ATP}-channel activity in cardiac cells[17], 0.5 mM Mg^{2+} alone had little if any effect on the activity of these channels in skeletal muscle at a pH_i of either 8.0 or 5.7 (see also ref. 9).

The relationship between P_{open} and pH_i in the presence and absence of Mg^{2+} and ATP at a membrane potential of −3 mV is shown in Fig. 3. In the presence of Mg^{2+} and ATP, P_{open} increased markedly with decreasing pH_i, whereas in the absence of ATP, P_{open} remained virtually constant. This was also seen in patches held at other membrane potentials. In two patches at −33 mV, the mean fractional current remaining after application of 1 mM $MgCl_2$ and 0.5 mM ATP was 0.28 at pH_i 5.7, whereas the mean of four patches at pH_i 7.2 was only 0.01. In another patch held at+27 mV, the fractional current remaining at pH_i 5.7 was 0.52 compared with 0.04 at pH_i 8.0. The activity of K_{ATP} channels was therefore highly sensitive to changes in pH_i in the presence of Mg^{2+} and ATP at all the membrane potentials studied. The most sensitive region was between pH_i 7.2 and 6.0, which is within the physiological range for skeletal muscle[13,14,18]. Almost all other types of K^+ currents, including inward and delayed rectifier currents, and Ca^{2+}-activated K^+ currents, are decreased by a fall in pH_i (refs 19-21). A decrease in pH_i has been shown to cause a reduction in the activity of K_{ATP} channels in pancreatic β-cells, although only in the presence of ATP[22]. But the K_{ATP} channels of skeletal muscle are unusual, as in the presence of Mg^{2+} and ATP, both of which are constituents of intracellular solution, a decrease in pH_i leads to an increase in the average current flowing through the channels. These results indicate that intracellular pH is important in regulating P_{open} of K_{ATP} channels in skeletal muscle. It will be interesting to see whether such an effect also occurs in cardiac and smooth muscle. □

Received 6 June; accepted 24 November 1989.

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ACKNOWLEDGEMENTS. I am grateful to Professor P. R. Stanfield and Dr N. B. Standen for their constructive criticism during the preparation of this manuscript, and the MRC for financial support.

Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases

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THE critical pathways through which protein-tyrosine kinases induce cellular proliferation and malignant transformation are not well defined. As microinjection of antibodies against $p21^{ras}$ can block the biological effects of both normal and oncogenic tyrosine kinases, it is likely that they require functional $p21^{ras}$ to transmit their mitogenic signals[1,2]. No biochemical link has been established, however, between tyrosine kinases and $p21^{ras}$. We have identified a non-catalytic domain of cytoplasmic tyrosine kinases, SH2, that regulates the activity and specificity of the kinase domain[3-9]. The presence of two adjacent SH2 domains in the $p21^{ras}$ GTPase-activating protein (GAP)[6,10,11] indicates that GAP might interact directly with tyrosine kinases. Here we show that GAP, and two co-precipitating proteins of relative molecular masses 62,000 and 190,000 (p62 and p190) are phosphorylated on tyrosine

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in cells that have been transformed by cytoplasmic and receptor-like tyrosine kinases. The phosphorylation of these polypeptides correlates with transformation in cells expressing inducible forms of the v-*src* or v-*fps* encoded tyrosine kinases. Furthermore, GAP, p62 and p190 are also rapidly phosphorylated on tyrosine in fibroblasts stimulated with epidermal growth factor. Our results suggest a mechanism by which tyrosine kinases might modify p21^{ras} function, and implicate GAP and its associated proteins as targets of both oncoproteins and normal growth factor receptors with tyrosine kinase activity. These data support the idea[4–6] that SH2 sequences direct the interactions of cytoplasmic proteins involved in signal transduction.

Rabbit antisera were raised against a trpE-GAP bacterial fusion protein containing amino-acid residues 171–448 from the N-terminal region of human GAP (ref. 11). Rat or mouse fibroblast cell lines transformed by the v-*fps*, v-*src* or v-*abl* retroviral oncogenes, which encode membrane-associated cytoplasmic tyrosine kinases (P130$^{gag\text{-}fps}$, p60$^{v\text{-}src}$ and P120$^{gag\text{-}abl}$ respectively), were lysed and immunoprecipitated with anti-GAP(171–448) antiserum. Western blotting of the precipitated proteins with affinity-purified anti-phosphotyrosine antibodies identified three polypeptides from the transformed cells,with mobilities corresponding to M_rs of 62,000 (62K), 124K and 190K (Fig. 1*a*). The 62K band was most prominent in each case, and migrated as a doublet in v-*src* and v-*abl*-transformed cells. Although the same phosphotyrosine-containing proteins were present in anti-GAP(171–448) immunoprecipitates from the different transformed cells, their relative abundance varied. In particular, tyrosine phosphorylation of the 124K protein, subsequently shown to be GAP, was highest in v-*src*-transformed Rat-2 cells (Rat-2 v-*src*). Immunoprecipitation of Rat-2 v-*src* cells with anti-peptide antibodies directed against residues 975–990 of human GAP yielded a pattern of phosphotyrosine-containing proteins identical to that observed with anti-GAP(171–448) antiserum (fig. 1*b*). Phosphotyrosine-containing proteins precipitated by anti-GAP antiserum were specific to

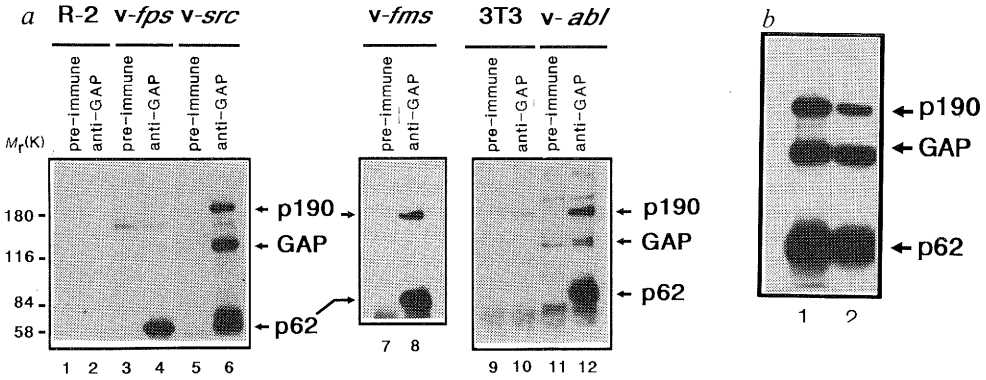

FIG. 1 GAP, p62 and p190 from cells transformed by tyrosine kinase oncoproteins are recognized by anti-phosphotyrosine antibodies. *a*, Immunoprecipitates from cell lysates using pre-immune serum (lanes 1, 3, 5, 7, 9, 11) or anti-GAP(171–448) antiserum (lanes 2, 4, 6, 8, 10, 12), were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with affinity-purified rabbit anti-phosphotyrosine antibodies followed by ^{125}I-protein A. Immunoprecipitates were from normal Rat-2 cells (lanes 1 and 2), Rat-2 cells transformed by avian v-*fps* (lanes 3 and 4), avian v-*src* (lanes 5 and 6) or feline v-*fms* (lanes 7 and 8), NIH 3T3 cells (lanes 9 and 10) or v-*abl*-transformed NIH 3T3 cells (lanes 11 and 12). *b*, Rat-2 v-*src* cells were immunoprecipitated with (lane 1) anti-GAP(171–448) or (lane 2) anti-GAP(975–990) antibodies, and immunoblotted with anti-phosphotyrosine antibodies.

METHODS. To obtain anti-GAP(171–448) antiserum, a *Ball*–*Sca*I restriction fragment from a human GAP complementary DNA was subcloned into the pATH11 bacterial expression vector. The 66 K trpE-GAP protein was induced, purified, and used to immunize rabbits[20]. Proteins were immunoprecipitated as detailed elsewhere[18]. Briefly, cells were lysed on ice in 1 ml 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM PMSF, 200 μM sodium orthovanadate, 10 mM pyrophosphate and 100 mM sodium fluoride. As minor fractions of the highly phosphorylated Gag-containing tyrosine kinases tend to precipitate non-specifically under these lysis conditions, pre-immune serum was always included as a negative control. About 2 × 10^6 cells were used in each immunoprecipitation, except for samples run in lanes 7 and 8, where ~1 × 10^7 cells were used. Lysates were centrifuged for 30 min at 10,000*g* and the supernatants incubated for 90 min at 4 °C with anti-GAP antiserum or pre-immune serum and 100 μl 20% protein A-Sepharose. The resulting immune complexes were washed three times with 20 mM HEPES, pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate, heated in SDS sample buffer, separated by gel electrophoresis, and immunoblotted with anti-phosphotyrosine antibodies[6,21]. The isolation and specificity of these antibodies has been described[6,21,22].

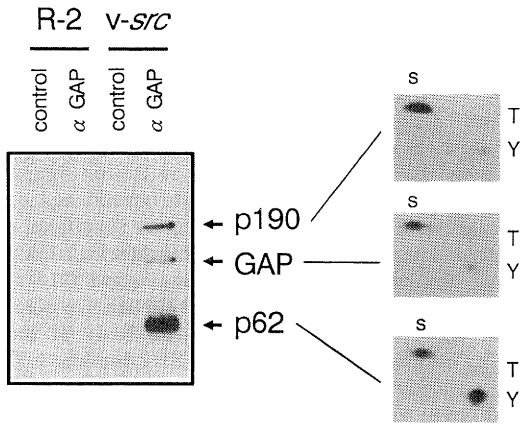

FIG. 2 Phosphorylation of GAP, p62 and p190 is induced by v-*src* transformation. Rat-2 cells and Rat-2 v-*src* cells were metabolically labelled with $^{32}P_i$, immunoprecipitated with control rabbit antibodies or affinity-purified anti-GAP(171–448) antibodies, separated by gel electrophoresis, transferred to an Immobilon membrane and autoradiographed (on the left). Phosphorylated GAP, p62 and p190 from Rat-2 v-*src* cells were analysed for phosphoamino acids (on the right). S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

METHODS. 5 × 10^6 Rat-2 cells or Rat-2 v-*src* cells in 10-cm plates were incubated for 2 h in phosphate-free Dulbecco's modified Eagle medium containing 2% dialysed fetal bovine serum and 5 mCi $^{32}P_i$. Lysates were prepared as described in the legend to Fig. 1, and were precleared once by incubation for 1 h with protein-A agarose beads coated with normal rabbit serum, and again by incubation with protein-A agarose beads coated with 10 μg affinity-purified rabbit anti-mouse immunoglobulin antibodies. The precleared lysates were divided into two and incubated with protein-A agarose beads coated with either 2 μg rabbit anti-mouse immunoglobulin antibodies (as a control), or with 2 μg affinity-purified rabbit anti-GAP(171–448) antibodies. The immunoprecipitates were washed and resolved by gel electrophoresis as for Fig. 1, electrophoretically transferred to an Immobilon membrane (Millipore) and exposed to XAR film at −70 °C for 10 h with an intensifying screen. Regions of the membrane containing the indicated ^{32}P-labelled proteins were incubated with 5.7 N HCl at 110 °C for 1 h[23]. The resulting hydrolysates were analysed by two-dimensional thin-layer electrophoresis[24] and exposed to film for 85 h with an intensifying screen.

the transformed cells, with the exception of p190, which was detectable at low levels in parental fibroblasts (Fig. 1*a*).

Metabolic labelling of normal or v-*src*-transformed Rat-2 cells with $^{32}P_i$ followed by immunoprecipitation with anti-GAP antibodies, showed that the total phosphorylation of GAP, p62 and p190 was strongly induced in the Rat-2 v-*src* cells (Fig. 2). Phosphoamino-acid analysis indicated that 65% of the phosphate in p62 from Rat-2 v-*src* cells was in the form of phosphotyrosine, compared with 14% for GAP and 7% for p190 (Fig. 2). Therefore p62 is highly phosphorylated on tyrosine in transformed cells, whereas the stoichiometry of GAP tyrosine phosphorylation seems to be low (Fig. 2; and unpublished results). But, the molecules of GAP containing phosphotyrosine could represent a functionally important subpopulation. All three proteins from transformed cells contained both phosphoserine and phosphothreonine, in addition to phosphotyrosine, and indeed phosphoserine was the chief phosphoamino acid of GAP and p190.

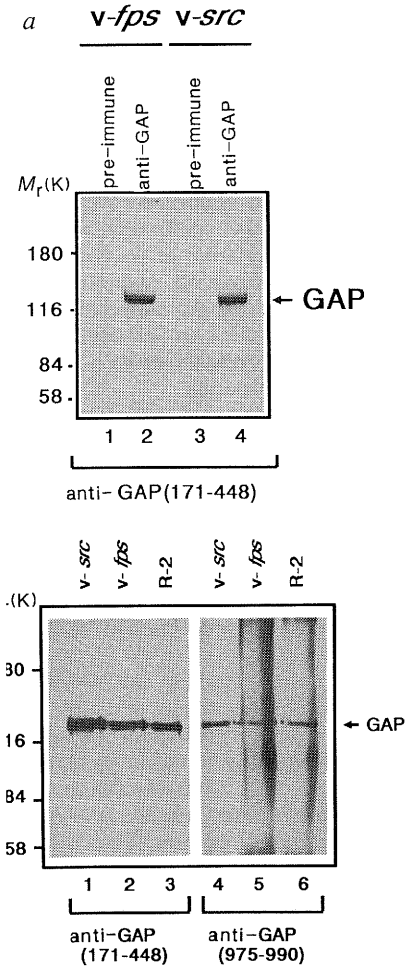

FIG. 3 The 124 K protein recognized by anti-phosphotyrosine antibodies is GAP. *a*, Immunoprecipitates obtained with pre-immune serum (lanes 1 and 3) or anti-GAP(171–448) antiserum (lanes 2 and 4) from Rat-2 cells transformed by v-*fps* (lanes 1 and 2) or v-*src* (lanes 3 and 4) were first blotted with anti-phosphotyrosine antibodies (Fig. 1*a*, lanes 3–6). The nitrocellulose was then stripped of antibody and reprobed with anti-GAP(171–448). *b*, Rat-2 cells transformed with v-*src* (lanes 1 and 4) or v-*fps* (lanes 2 and 5), or parental Rat-2 cells (lanes 3 and 6) were lysed and immunoprecipitated with anti-GAP(171–448) antiserum. Precipitates were immunoblotted directly with anti-GAP(171–448) antiserum (lanes 1–3) or affinity-purified anti-GAP(975–990) antibodies (lanes 4–6). Migration of molecular weight calibration standards is shown on the left ($\times 10^{-3}$).

METHODS. In *a*, anti-phosphotyrosine antibodies were stripped from the western blot by two successive 15 min incubations with a solution containing 5 M sodium iodide and 1 mM sodium thiosulphate, followed by a brief rinse in distilled water. After autoradiography to confirm that all radioactivity was lost, the stripped blot was reprobed with anti-GAP(171–448) antiserum. In both *a* and *b* bound antibody was identified using an alkaline phosphatase-conjugated secondary antibody, followed by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate[6].

The phosphotyrosine-containing proteins precipitated by anti-GAP(171–448) antiserum were blotted with the same antiserum, or with antibodies to the GAP(975–990) peptide (Fig. 3). Both anti-GAP antibodies recognized only a single main band, the mobility of which was identical to that of 124K protein previously identified with anti-phosphotyrosine antibodies in transformed cells (compare Fig. 1*a*, lanes 3–6, with Fig. 3*a*). These results confirm that the 124K protein phosphorylated on tyrosine in cells transformed by v-*fps*, v-*src* or v-*abl* is GAP itself. The expression of GAP, in contrast to its phosphorylation, was equivalent in both normal and transformed cells (Fig. 3*b*). Potentially, p62 and p190 could be precipitated because they are complexed with GAP, or could be recognized directly by anti-GAP antibodies. As p62 and p190 were precipitated by antibodies both to the N- and the C-terminal regions of GAP, but were not recognized by either antibody on immunoblots, direct antibody binding seems unlikely. In anti-GAP immunoprecipitates of [^{35}S]methionine-labelled v-*src*-transformed cells, p190 was readily identified but it was not found in immunoprecipitates of normal Rat-2 cells. Boiling lysates of transformed cells in 0.5% SDS abolished the subsequent immunoprecipitation of p190 and p62, but not of GAP (data not shown). We conclude that p190 and p62 form a specific complex with GAP. As p62 did not seem to bind either antibody on a western blot, it is unlikely to be a proteolytic fragment of GAP, and indeed it has a distinct tryptic phosphopeptide map (data not shown).

To extend these observations to receptor-like tyrosine kinases, we first examined Rat-2 cells transformed by v-*fms*, which encodes an activated form of the macrophage colony stimulating factor receptor[12] (Fig. 1*a*). Anti-GAP immunoprecipitates from these cells contained tyrosine-phosphorylated p62 and p190, although about fivefold more cells were required to generate a signal equivalent to that observed with the cytoplasmic oncoproteins. GAP from v-*fms*-transformed cells was poorly reactive with anti-phosphotyrosine antibodies, but could be clearly detected after prolonged exposure. These observations suggest that transmembrane as well as cytoplasmic tyrosine kinases interact directly with GAP. To investigate GAP phosphorylation after growth factor stimulation we used Rat-1 fibroblasts expressing $\sim 2.5 \times 10^5$ human epidermal growth factor (EGF)-receptors per cell (W. Wasilenko and M. Weber, personal communication). EGF-treatment of starved cells induced phosphorylation of p62, GAP and p190 within 2.5 min (Fig. 4*a*), suggesting that they are substrates for the EGF-receptor. There was a similar rapid phosphorylation of GAP and GAP-associated proteins after stimulation of Rat-2 cells with platelet-derived growth factor (data not shown). Phosphorylation of GAP, p62 and p190 is therefore a feature of the normal signal transduction process in fibroblasts.

We used two cell lines that are inducible for v-*fps* or v-*src* transforming activity to investigate the relationship between the phosphorylation of GAP and GAP-associated proteins and transformation. The addition of heavy metals to a Rat-2 cell line that contains the coding sequence for P130$^{gag\text{-}fps}$ under the control of the human metallothionein-IIA promoter, induces a 14-fold increase in P130$^{gag\text{-}fps}$ expression over the course of 24 h, resulting in their conversion from a normal to a transformed phenotype (M.M., unpublished results). Untreated cells, or cells collected after 24 h of heavy-metal treatment, were immunoprecipitated with an anti-Gag monoclonal antibody (which specifically recognizes P130$^{gag\text{-}fps}$) or with anti-GAP antiserum, and the immunoprecipitates were blotted with anti-phosphotyrosine antibodies (Fig. 4*b*) The basal level of tyrosine phosphorylation is higher in these cells than in normal Rat-2 cells owing to residual v-*fps* expression in the absence of zinc

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ACKNOWLEDGEMENTS. We thank Theresa Lee and Laurie Gray for technical assistance, Peter Greer and Gareth Taylor for advice and help with bacterial expression and immunization, Kenneth Letwin for anti-phosphotyrosine antibodies and Anne Koch for cell lines. We are indebted to Michal Weber for Rat-1 cell lines expressing the human EGF-receptor and tsLA29 *src*, and to Jeff Kudlow for EGF. This work was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada.

Immunological activity of covalently linked T-cell epitopes

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IMMUNE responses to proteins necessarily involve the recognition by T lymphocytes of a peptide or peptides derived from a protein complexed with a major histocompatibility antigen. The T-cell response of BALB/c mice to the bacteriophage λ cI repressor protein (residues 1–102) is directed predominantly towards the epitope contained within a single peptide encompassing residues 12–26 (refs 1, 2). Similar phenomena of immunodominance of a particular peptide have also been observed in other protein systems[3–6]. The mechanisms that have been suggested to account for the focusing of the T-cell response[7] are partial deletion in the T-cell repertoire, biased antigen processing, and competition for binding to the presenting molecule, the major histocompatibility complex encoded class II transplantation antigen. In a model system with a polypeptide containing two synthetically linked immunologically active epitopes, we now demonstrate the existence of a hierarchy between these epitopes, so that the immune response elicited is directed mainly towards the more immunogenic epitope, whereas the less immunogenic epitope elicits little or no T-cell reactivity. In addition, the same hierarchy of dominance is also apparent when the polypeptide is used to induce tolerance in the periphery in adult mice. The chimaeric peptide can induce tolerance only towards the more immunogenic epitope. These experiments indicate that the rules governing antigen processing and presentation that result in T-cell activation are apparently the same as the rules that govern the processes resulting in the induction of tolerance.

We have synthesized a peptide containing residues 12–26 of the λ repressor protein cI (p12–26), joined through a Gly-Pro-Gly sequence to residues 325–336 of chicken ovalbumin (p325–336). This joined peptide contains the immunodominant epitopes of the two proteins expressed in BALB/c mice[1,6]. The ovalbumin-derived peptide was modified at position 327 by the substitution of valine by aspartic acid (pOVAD). In confirmation of previous results[8], this single amino-acid substitution decreased the apparent affinity of the p325–336 peptide for the I-A^d-encoded class II major histocompatibility complex (MHC) molecule (Fig. 1*d*), whereas the epitope recognized by the T-cell receptor within this peptide was apparently unaltered. The two distinct T-cell epitopes, p12–26 and pOVAD, in the joined peptide were each immunologically active *in vitro* (Fig. 1*a–c*). The joined peptide was able to stimulate both the λ repressor cI p12–26-specific T-cell hybridomas 9C127 and 1E1, and the ovalbumin-specific hybridoma 3DO54.8. Depending on the

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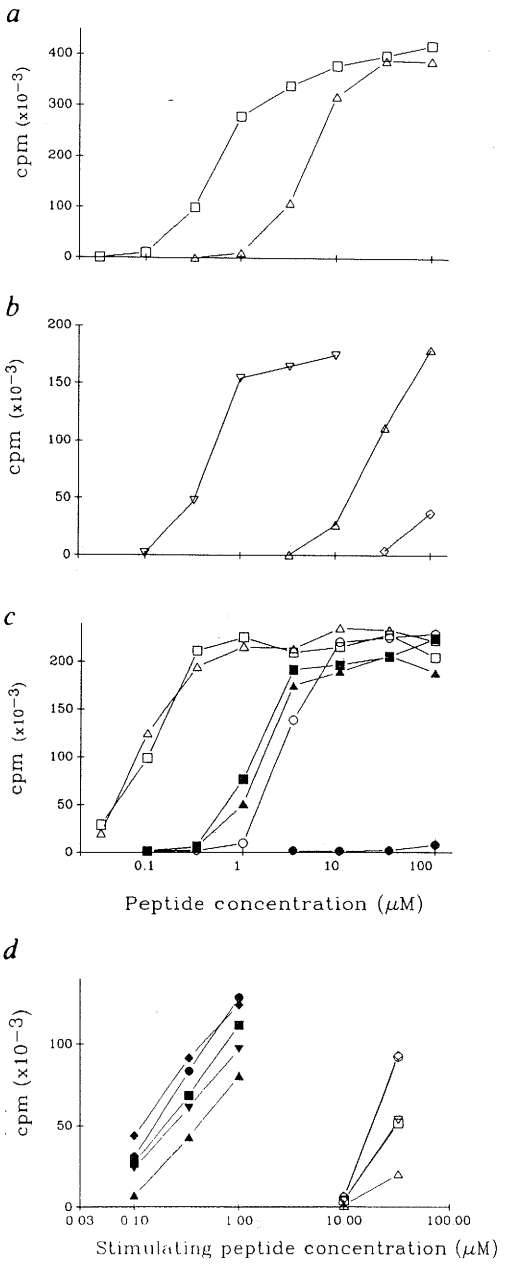

FIG. 1 *a, b* and *c, In vitro* activity of the joined peptide (p12–26-Gly-Pro-Gly-pOVAD). The ability of the joined peptide to stimulate λ repressor cI (12–26) and ovalbumin-specific hybridomas was tested using the hybridomas 9C127 (*a*), eDO54.8 (*b*) and 1E1 (*c*). The hybridomas were stimulated with the joined peptide (△), p12–26 (□), pOVAD (◇), pOVAD(324–336; ▽), p1–102 of λ repressor protein cI (○). As antigen-presenting cells, L cells (*a, b*) and A20 (open symbols) or fixed A20 (closed symbols; *c*) were used. *d*, Ability of pOVAD (open symbols) and pOVA (324–336; closed symbols) to stimulate the 3DO54.8 hybridoma when presented alone (●) or in presence of different concentrations of p12–26 (100 μM (▲), 50 μM (■), 25 μM (▼), 12.5 μM (◆)). A20 cells were used as antigen-presenting cells.
METHODS. Details of the preparation of the p12–26-specific hybrodimas are described elsewhere[16]. The hybridoma 3DO54.8 was kindly provided by Drs J. Kappler and P. Marrack. The L cells RT 2.3.3.H, expressing I-A^d-encoded molecule, were a gift of Dr R. N. Germain. The complete sequence of λ repressor protein is reported by Sauer and Andregg[17]. All peptides were synthetized by the solid phase method of Merifield[18] using an automated Applied Biosystem 430A peptide synthesizer. The hybridomas (5×10^4 cells per well) were stimulated by APC cells (5×10^4 cells per well) in the presence of varying concentrations of test peptides. After 24 h, interleukin-2 secretion was measured previously described[16]. APC cells (A20) were fixed with 0.1% glutaraldehyde in PBS, and the reaction was quenched with 0.2 M lysine in PBS after 30 s.