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An interaction between p21^{ras} and heat shock protein hsp60, a chaperonin

(oncogenes/signal transduction/molecular chaperon)

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ABSTRACT **Ras proteins play a crucial role in the development of neoplasia and in signal transduction in normal cells. In a search for proteins interacting with p21^{ras}, we previously identified a protein of 60 kDa (p60) through use of a chemical cross-linker. Using information from partial amino acid sequencing of the purified protein, we isolated full-length cDNA clones encoding this 60-kDa protein. Nucleotide sequence analysis revealed that p60 is the murine heat shock protein hsp60, a chaperonin. Association of hsp60 with p21^{ras} appears physiological, as the amount of hsp60 complexed to p21^{ras} was similar even in cells over-expressing p21^{ras}, and reversing the order of cross-linking and lysis of the cells, which releases large amounts of hsp60 from mitochondria, did not alter the amount of hsp60 cross-linked to p21^{ras}.**

The Ha-, Ki-, and N-*ras* oncogenes have attracted the attentions of those interested in carcinogenesis since the discovery of their point-mutated forms in human tumors (1). p21 proteins encoded by the normal *ras* alleles appear to play a role in the cell transformation induced by various tyrosine kinase oncogenes and in the cell proliferation triggered by various growth factors (2–4).

The sequence similarity of Ras and G proteins suggests that Ras proteins function as signal-transducing molecules (5, 6). Like G proteins, p21^{ras} cycles between a GTP- and a GDP-bound form. Oncogenic mutations either impair its intrinsic GTPase activity or accelerate the rate of GTP/GDP exchange. As a consequence, a net increase in the p21^{ras} population loaded with GTP is achieved (2–4). Furthermore, the proportion of GTP-bound p21^{ras} increases after exposure to certain growth stimuli (7–10). Taken together, these data suggest that the GTP-bound p21^{ras} represents its activated form that emits growth-stimulatory signals.

At least two proteins might be expected to interact with p21^{ras}: a molecule sending an afferent signal to p21^{ras} (activator-exchange protein) and a molecule receiving an efferent signal from p21^{ras} (effector). In recent years, a number of proteins have been identified that are implicated in direct interactions with p21^{ras} in yeast and in mammals (2, 11). In mammals, the proteins interacting with p21^{ras} may be placed into two groups: those that potentiate the GTPase activity of p21^{ras}, such as GTPase-activating protein (GAP) and the locus involved in hereditary neurofibromatosis (NF-1) (12–14) and those that catalyze GTP/GDP exchange by p21^{ras} (15–18). Nevertheless, their role in the *ras* pathway is still unclear (2, 9, 19).

Workers in our laboratory have identified yet another protein, termed p60, which also appears to interact directly with p21^{ras} (20). This protein was identified by using the chemical cross-linker dithio-bis(succinimidyl propionate) (DSP). DSP is a membrane-permeant chemical ideal for cross-linking proteins within living cells. p60 was a good candidate for an effector molecule because the proportion of p21^{ras} bound to p60 was ≈5%, which increased 2- to 3-fold upon serum stimulation (20), comparable to the proportion of p21^{ras} loaded with GTP in fibroblasts (8, 10). Accordingly, we undertook the task of cloning the gene encoding p60. In this report, we describe the identity of p60 and discuss its possible significance for *ras* functioning.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. 70z cells were grown in RPMI 1640 medium/10% Nu-serum (Collaborative Research)/50 μM 2-mercaptoethanol. Rat-1 cells and their derivatives over-expressing Ras proteins (21) were grown in Dulbecco's modified Eagle's medium (DMEM)/10% calf serum. Jurkat cells were grown in RPMI medium/10% fetal calf serum.

Antibodies. Monoclonal antibody (mAb) 147 was generated against a peptide corresponding to residues 157–180 of p21$^{c\text{-}K\text{-}ras4A}$. The hybridoma producing mAb 147 [National Cancer Institute Repository (Microbiological Associates)] was adapted to grow serum-free in hybrid medium (RPMI medium/DMEM, 1:1)/Nutridoma-SP (Boehringer Mannheim)/10 μM 2-mercaptoethanol/0.5% glucose. The culture supernatant was concentrated and purified using a protein G column (Pierce). Anti-Ha-Ras sera were from J. deGunzburg; these were rabbit antisera raised against p21$^{c\text{-}H\text{-}ras}$ overproduced in *Escherichia coli* as described (20). ML-30, a mAb for *Mycobacterium leprae* 65-kDa antigen that also recognizes mammalian hsp60 (22), was from T. Schinnick (Centers for Disease Control, Atlanta).

Labeling and Chemical Cross-Linking Conditions. Subconfluent cultures were labeled for 4–6 hr with 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml (1200 Ci/mmol; Tran^{35}S label; ICN) in methionine-free medium/10% dialyzed fetal calf serum. Cross-linking and lysis were done as described (20) with three modifications. (*i*) Lysis buffer was modified to 50 mM Hepes, pH 7.4/0.1 M NaCl/1 mM EGTA/1% Triton X-100 supplemented with protease inhibitors as described (20). (*ii*) After cross-linking, DSP was quenched by adding ammonium acetate to a final concentration of 50 mM. (*iii*) The cross-linking step and cell lysis step were reversed where indicated.

Immunoprecipitation and Analyses of the Products. Immunoprecipitation and analyses of the products were done as described (20), except that immunoprecipitation with mAb 147 required an additional incubation with 3 μg of rabbit anti-mouse IgG (Cappel Laboratories) because mAb 147 was of the mouse IgG1 subclass. Immunoblotting was done by using the Protoblot kit (Promega) according to the manufacturer's instructions.

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Abbreviations: mAb, monoclonal antibody; DSP, dithio-bis(succinimidyl propionate).

Purification of the p60. Fifty liters of 70z cells was collected, washed twice in phosphate-buffered saline (PBS), and incubated 30 min on ice with 1 mM DSP/PBS (1 liter) freshly prepared from 0.1 M DSP in dimethyl sulfoxide. Ammonium acetate was then added to 50 mM final concentration, cells were washed twice with PBS and lysed with 600 ml of lysis buffer, and debris was removed by centrifugation for 30 min at 20,000 × *g*. The lysate was incubated with 5 mg of mAb 147 for 24 hr, with 6 mg of immunopurified rabbit anti-mouse IgG (Cappel Laboratories) for 24 hr, and with Affi-Gel protein A (Bio-Rad) for 24 hr with gentle rotation at 4°C. After further washes as described (20), proteins were eluted with 6 ml of 10 mM Tris·HCl, pH 7.5/5 mM EDTA/0.2 M dithiothreitol for 30 min at 37°C. Proteins were concentrated with a Centricon 30 filter (Amicon) and fractionated by electrophoresis through a 8.5% SDS/polyacrylamide gel. Proteins were then transferred to a nitrocellulose filter and stained with Ponceau-S; the band corresponding to the p60 was then excised as described (23).

Microsequencing of p60. *In situ* tryptic digestion on a nitrocellulose filter, purification of the resulting peptides, and microsequencing of the peptides were done by W. F. Lane (Harvard Microchemistry Facility, Harvard University).

Cloning and Sequencing of the p60 Gene. A 23-mer oligopeptide sequence (QALLDAAGVASLLTTAEAVVVE) was the longest obtained by microsequencing. Consequently, two oligonucleotide corresponding to its two ends were synthesized: CCCGAATTCA(R)GCN(Y)TN(Y)T-NGA(Y)GC (1024-fold degenerate) corresponding to its amino-terminal 6 amino acids plus an *Eco*RI linker and CCCG-GATCCTCNCANTGNTGNCG(R)AGNCG (512-fold degenerate) corresponding to its carboxyl-terminal 6 amino acids plus a *Bam*HI linker. PCR was done by using these as primers, and cDNA was synthesized from the 70z cell mRNA as a template. The resulting amplified DNA fragment was digested with *Eco*RI and *Bam*HI and cloned into the pTZ18U vector (United States Biochemical). The insert was used as a probe for screening a λgt110 cDNA library constructed from the mouse pre-B cell line 22D6 [from M. A. Oettinger (Massachusetts General Hospital), D. G. Schatz (Yale Medical School), and D. Baltimore (The Rockefeller University)]. The sequence analysis was done by using the Sequenase kit version 2.0 (United States Biochemical) after the construction of nested deletion plasmids with the Nested Deletion kit (Pharmacia) according to the manufacturer's instructions.

RESULTS

Purification of p60. We performed a pilot purification to show that the p60 that we purified on a small scale was identical to the p60 identified and characterized previously (20). Thus, a 6-liter culture of 70z cells and 10^7 cells of metabolically labeled 70z cells were mixed, treated with DSP cross-linker, and lysed. The $p21^{ras}$–p60 complex was recovered by immunoprecipitation using an excess of mAb 147 (a mAb that recognizes specifically $p21^{K\text{-}ras4A}$), rabbit anti-mouse IgG, and protein A-agarose beads. Material released after cleavage of the cross-linker with dithiothreitol was fractionated by SDS/PAGE followed by silver staining and direct autoradiography of the same gel (Fig. 1).

Bands in the silver-stained gel (Fig. 1*B*) should represent the p60 to be purified, whereas bands in the autoradiograph (Fig. 1*A*) should represent the p60 identified and characterized previously (20). The resulting bands in the silver-stained gel and the autoradiograph were similar, except for IgG bands released from the immune complex that appeared in the silver-stained gel. The p60 bands only appeared in cross-linked cells immunoprecipitated with mAb 147 (Fig. 1 *A* and *B*, lane 6) and disappeared when mAb 147 was preincubated with its cognate antigen oligopeptide (Fig. 1 *A* and *B*, lane 5),

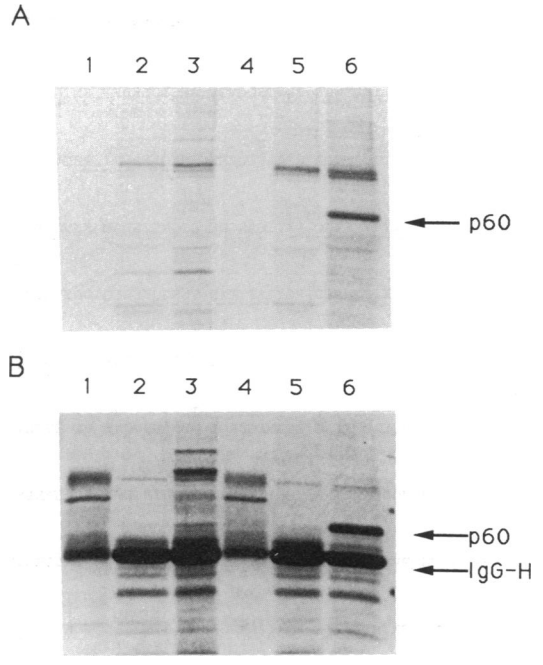

FIG. 1. Analysis of purified p60 on small scale. p60 purified from a mixture of unlabeled and ^{35}S-labeled 70z cell lysate was analyzed by direct autoradiography (*A*) and silver staining (*B*). The autoradiograph was obtained by direct exposure of the silver-stained gel. Lysates from uncross-linked cells (lanes 1–3) and cross-linked cells (lanes 4–6) were immunoprecipitated with normal mouse serum (lanes 1 and 4), mAb 147 absorbed with its cognate peptide (lanes 2 and 5), and mAb 147 (lanes 3 and 6), respectively. Proteins eluted by dithiothreitol were analyzed. Arrows indicate positions of p60 and IgG heavy chain (IgG-H).

suggesting that p60 purified by this method was, indeed, identical to the p60 identified earlier (20).

We sought to optimize the conditions for immunoprecipitation by altering the ratios of lysate, mAb 147, second antibody, and protein A-agarose beads. The optimized ratio was 100 μg of mAb 147, 120 μg of second antibody, and 120 μl of protein A-agarose beads per lysate prepared from a liter of 70z cells (≈10^9 cells), yielding 200 ng of p60. We directly scaled up this protocol and applied it to a lysate prepared from a 50-liter culture of 70z cells. In so doing, we obtained ≈10 μg of p60. This p60 preparation was transferred to a nitrocellulose filter, excised, and sent to W. F. Lane (Harvard University) for microsequencing.

Cloning of p60. Using the information obtained by microsequencing and the approach described, we obtained eight phages out of 10^5 plaques from a cDNA library, all carrying 2.3-kilobase (kb) cDNA inserts. Because the mRNA of the mouse p60 gene was subsequently determined to be ≈2.4 kb by Northern (RNA) blot hybridization (data not shown), we concluded that these phages carry full-length cDNA clones of this gene.

One of these 2.3-kb cDNA inserts was subjected to sequence analysis, and the amino acid sequence of its encoded protein was deduced (Fig. 2). Comparison of this sequence with those in the data base (Protein Identification Resource, National Biomedical Research Foundation) revealed that the p60 encoded by this cDNA is hsp60, a heat shock protein (24, 25). hsp60 is known to be an abundant cellular protein localized largely to the mitochondrial matrix. Its functions are reported to involve aiding the proper folding and proper positioning of other proteins found in multimeric complexes (i.e., a chaperonin) (26, 27).

This identification of p60 with hsp60 was strengthened by use of ML-30 antibody. ML-30 is a mAb generated against the

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human     L   F          V
mouse  MLRHPTVLRQ MRPVSRALAP HLTRAYAKDV KFGADARALM LQGVDLLADA  50

human
mouse  VAVTMGPKGR TVIIEQSWGS PKVTKDGVTV AKSIDLKDKY KNIGAKLVQD 100

human                                D             E
mouse  VANNTNEEAG DGTTTATVLA RSIAKEGFEK ISKGANPVKI RRGVMLAVDA 150

human                                    E
mouse  VIAELKKQSK PVTTPEEIAQ VATISANGDK DIGNIISDAM KKVGRKGVIT 200

human
mouse  VKDGKTLNDE LEIIEGMKFD RGYISPYFIN TSKGQKCEFQ DAYVLLSEKK 250

human     I
mouse  ISSVQSIVPA LEIANAHRKP LVIIAEDVDG EALSTLVLNR LKVGLQVVAV 300

human                                    T       P
mouse  KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL NLNLEDVQAH DLGKVGEVIV 350

human                  Q         I   V
mouse  TKDDAMLLKG KGDKAHIEKR IQEITEQLDI TTSEYEKEKL NERLAKLSDG 400

human
mouse  VAVLKVGGTS DVEVNEKKDR VTDALNATRA AVEEGIVLGG GCALLRCIPA 450

human      T                 T                          M
mouse  LDSLKPANED QKIGIEIIKR ALKIPAMTIA KNAGVEGSLI VEKILQSSSE 500

human         A                                          V
mouse  VGYDAMLGDF VNMVEKGIID PTKVVRTALL DAAGVASLLT TAEAVVTEIP 550

human
mouse  KEEKDPGMGA MGGMGGGMGG GMF* 573
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FIG. 2. Deduced amino acid sequence of p60 (= hsp60). Deduced amino acid sequence of p60 is aligned with the published sequence of human hsp60. For human hsp60, only different amino acid residues are shown. Solid line represents mitochondrial targeting sequence, broken lines represent sequence identified by partial amino acid sequencing of the purified p60, and double broken line represents the 23 amino acid sequence used for cloning of p60-encoding gene.

65-kDa antigen of *M. leprae* (hsp60). hsp60 is so well conserved that this ML-30 antibody also recognizes mammalian hsp60 (22). When the ML-30 antibody was used in immunoblotting analysis, it detected hsp60 in a cross-linked complex from 70z cells that was immunoprecipitated either with mAb 147 (Fig. 3*A*) or in a cross-linked complex from Rat-1 cells that was immunoprecipitated with polyclonal rabbit serum elicited against bacterially produced $p21^{H\text{-}ras}$ (anti-Ha-ras serum; data not shown), indicating that the p60 associated with $p21^{ras}$ is, indeed, hsp60.

Specificity of $p21^{ras}$–p60 Interaction. We undertook further experiments to rule out the possibility that the $p21^{ras}$–hsp60 association was nonspecific and driven by high concentrations of hsp60 within the cell. In fact, the great bulk of the hsp60 is associated with mitochondria (28, 29), whereas $p21^{ras}$ is found in a distinct intracellular compartment, the extra-mitochondrial cytoplasm (3, 4, 30).

With this in mind, we released hsp60 from its mitochondrial compartment by treatment of 70z cells with 1.0% of Triton X-100, a nonionic detergent, before treatment with the crosslinker. This reversal of the order of steps in our protocol did not cause any increase in the amount of hsp60 associated with $p21^{ras}$ (Fig. 3*A*, lanes 2 and 3). Accordingly, we conclude that the hsp60–$p21^{ras}$ association preexists in the cell before mitochondrial lysis. Moreover, the amount of this complex is not affected when the levels of free hsp60 are increased after its release from the mitochondria. Note that hsp60 cross-linked to $p21^{ras}$ migrates faster than hsp60 produced in *E. coli* (Fig. 3*A*), which may result from removal of the mitochondrial targeting sequence in mammalian cells (24, 31).

Further, the amounts of the complex immunoprecipitated with the anti-Ha-*ras* serum were not found to be greater when cross-linking was done with Rat-1 cells over-expressing a 97-fold (Fig. 3*B*, lanes 1 and 2) or a 36-fold (Fig. 3*B*, lanes 4 and 5) increased level of $p21^{H\text{-}ras}$ relative to their parental Rat-1 cells (Fig. 3*B*, lanes 7 and 8). This result was seen whether cross-linking was undertaken before (Fig. 3*B*, lanes 1, 4, and 7) or after (Fig. 3*B*, lanes 2, 5, and 8) cell lysis by nonionic detergent. (In this experiment, the background was higher from use of polyclonal rabbit serum.)

This absence of increased complex formation in cells vastly over-expressing $p21^{H\text{-}ras}$ could be explained by the fact that hsp60 only interacts with a subset of cellular $p21^{ras}$ proteins—namely, the $p21^{K\text{-}ras4A}$ studied in our earlier experiments. Thus, we sought evidence that p60 could associate with more than this one form of $p21^{ras}$. To do this, we used not only the

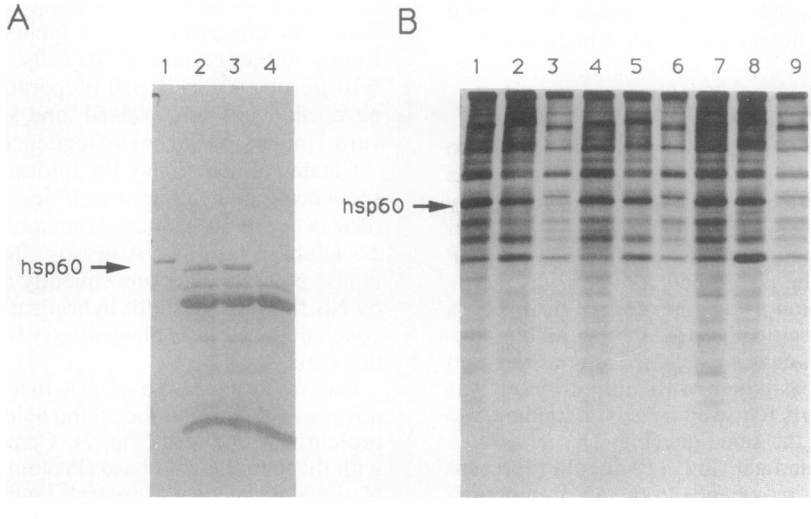

FIG. 3. Analysis of hsp60 bound to $p21^{ras}$ after reversal of cross-linking and cell lysis steps. (*A*) hsp60 recovered by using mAb 147 from 70z cells was analyzed by immunoblotting and probed with ML-30 (hsp60 antibody). Human hsp60 over-expressed in *E. coli* (lane 1), hsp60–$p21^{ras}$ immune complex precipitated from cross-linked cells before cell lysis (lane 2), from cross-linked cells after cell lysis (lane 3), and from uncross-linked cells (lane 4) are shown. (*B*) hsp60 recovered from Rat-1 cell derivatives metabolically labeled with ^{35}S was analyzed. $p21^{ras}$–hsp60 complex was immunoprecipitated with anti-Ha-ras serum from Rat-1 derivative cell clones over-expressing a 97-fold (lanes 1–3) or a 36-fold (lanes 4–6) increased level of $p21^{H\text{-}ras}$ relative to parental Rat-1 cells and from parental Rat-1 cells (lanes 7–9). hsp60–$p21^{ras}$ immune complexes were precipitated from cross-linked cells before cell lysis (lanes 1, 4, and 7), from cross-linked cells after cell lysis (lanes 2, 5, and 8), and from uncross-linked cells (lanes 3, 6, and 9).

$p21^{K\text{-}ras4A}$-specific mAb 147 but in addition the anti-Ha-ras polyclonal serum, which we determined was reactive with all types of $p21^{ras}$. We used these in sequential immunoprecipitations of lysates of metabolically labeled 70z cells treated with cross-linker (Fig. 4). If we used mAb 147 to precipitate $p21^{K\text{-}ras4A}$–hsp60 complex under the condition previously determined to yield >95% recovery of the complex (Fig. 4, lane 1), the anti-Ha-ras serum was still capable of precipitating hsp60–$p21^{ras}$ complex from the remaining lysate (Fig. 4, lane 2). Therefore, we conclude at least one other $p21^{ras}$ protein besides $p21^{K\text{-}ras4A}$ can bind to hsp60.

When we used lysates from metabolically labeled Jurkat cells, known to express $p21^{N\text{-}ras}$ and $p21^{K\text{-}ras}$ but not to express detectable $p21^{H\text{-}ras}$ (9) (S.I., unpublished result), for immunoprecipitation with the anti-Ha-ras serum, no bound hsp60 was detected (Fig. 5, lane 1), whereas, hsp60 was readily detectable by mAb 147 (Fig. 5, lane 5). We note in passing that a small amount of hsp60 was immunoprecipitated by anti-hsp60 antibodies present in normal rabbit serum (Fig. 5, lanes 1–4) (32). The intensities of hsp60 bands are stronger in cross-linked cells (Fig. 5, lanes 1 and 3) probably because hsp60 is a 14-mer complex (27), and hence one antibody molecule can precipitate 14 hsp60 monomers.

We conclude that the $p21^{ras}$–hsp60 complex detected by the anti-Ha-ras serum is, in fact, largely, if not exclusively, a $p21^{H\text{-}ras}$–hsp60 complex. Accordingly, hsp60 can bind to at least $p21^{K\text{-}ras4A}$ and $p21^{H\text{-}ras}$. Moreover, the absence of increased complex seen in the $p21^{H\text{-}ras}$-over-expressing cells cannot be ascribed to an inability of the anti-Ha-ras serum to recognize $p21^{H\text{-}ras}$–hsp60 complexes.

DISCUSSION

By use of an *in vivo* cross-linking procedure, we have identified a 60-kDa $p21^{ras}$-associated protein and found it to be identical to hsp60, a well-characterized chaperonin. This interaction does not appear artifactual due to cross-linking or a mass action effect driven by the abundance of hsp60. We conclude this for several reasons.

(*i*) Reversing the order of cross-linking and cell lysis did not alter the amount of hsp60 complexed with $p21^{ras}$. Because lysis of cell membranes releases large amounts of hsp60 from its mitochondrial reservoir, one would expect the amount of hsp60 linked to $p21^{ras}$ to be increased were this association from nonspecific aggregation. This result was not observed.

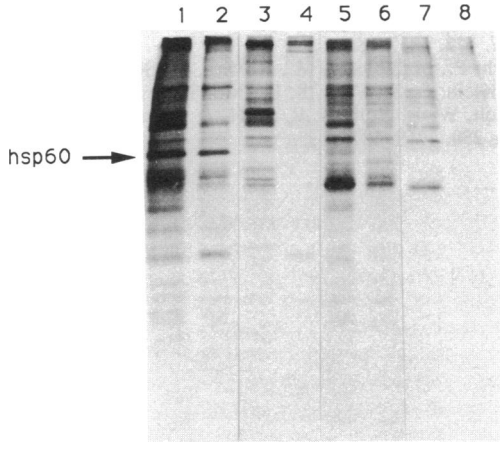

FIG. 4. Analysis of hsp60 cross-linked to $p21^{ras}$ by sequential immunoprecipitation with mAb 147 and anti-Ha-ras serum. $p21^{ras}$–hsp60 complex was immunoprecipitated from cross-linked (lanes 1–4) and uncross-linked (lanes 5–8) 70z cells metabolically labeled with ^{35}S. One lysate was immunoprecipitated first with mAb 147 (lanes 1 and 5), and the remaining supernatant was successively immunoprecipitated with anti-Ha-ras serum (lanes 2 and 6). Other lysates were immunoprecipitated with normal mouse serum (lanes 3 and 7) or with normal rabbit serum (lanes 4 and 8).

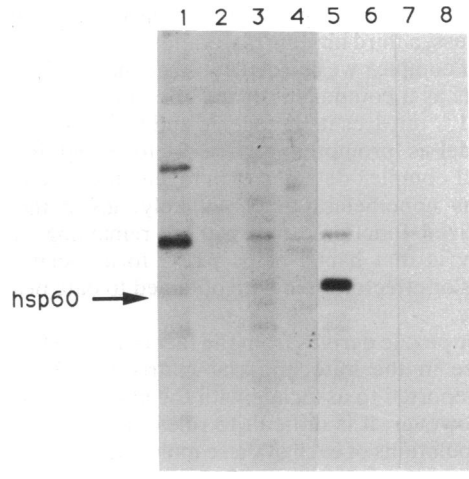

FIG. 5. Analysis of $p21^{ras}$–hsp60 complex in Jurkat cells. $p21^{ras}$–hsp60 complexes were immunoprecipitated from cross-linked (lanes 1, 3, 5, and 7) and uncross-linked (lanes 2, 4, 6, and 8) Jurkat cells metabolically labeled with ^{35}S with anti-Ha-ras serum (lanes 1 and 2), normal rabbit serum (lanes 3 and 4), mAb 147 (lanes 5 and 6), and normal mouse serum (lanes 7 and 8).

(*ii*) The complex of $p21^{ras}$ and hsp60 is precipitated by two different antibodies, mAb 147 and the anti-Ha-ras serum, recognizing two distinct Ras proteins, $p21^{K\text{-}ras4A}$ and $p21^{H\text{-}ras}$, respectively. Furthermore, multiple independent serum preparations from different rabbits immunized with bacterially produced $p21^{H\text{-}ras}$ recognized the complex (20), and hsp60 was not detectable with anti-Ha-ras serum in cross-linked Jurkat cells that do not express detectable $p21^{H\text{-}ras}$ (Fig. 5). In addition, recognition of the $p21^{ras}$–hsp60 complex by mAb 147 was inhibited by peptide encompassing residues 157–180 but not by $p21^{c\text{-}H\text{-}ras}$. Conversely, recognition of the complex by anti-Ha-ras sera was inhibited by added $p21^{H\text{-}ras}$ but not by the peptide (20) (S.I., unpublished result).

Not presented here are data showing that most anti-$p21^{ras}$ mAbs failed to recognize the complex in cross-linked cells (20) (S.I., unpublished result). This failure may well be explained by the fact that critical epitopes are being occluded or altered by $p21^{ras}$–hsp60 complex formation. $p21^{ras}$ cosedimented with the hsp60 fraction in a sucrose-gradient centrifugation experiment of the cross-linked cells (20), and the released materials from this fraction was recognized by various Ras antibodies, including Y13-259 and ras10 (33) (Jean deGunzburg, personal communication).

(*iii*) Even though hsp60 is an abundant protein, we did not find any increase in associated hsp60 by over-expressing $p21^{H\text{-}ras}$ up to 97-fold.

(*iv*) We think it unlikely that antibody reactive with hsp60 is contained in our antibodies (32). Thus, mAb 147 was purified from hybridoma supernatants prepared under serum-free culture conditions, eliminating the possibility of anti-hsp60 antibody contamination from serum. This possibility of adventitious cross-reactivity of the antibody was lessened further by the fact that mAb 147 failed to detect hsp60 from cells not exposed to the crosslinker.

For these reasons, we believe that the p21–hsp60 complexes detected by cross-linking appear to reflect a complex that exists in the cells before cross-linking. What might be the significance of this interaction? Because the function of hsp60 is to assist proper folding of proteins and the formation of protein complexes, one possibility is that this interaction may be related to the biogenesis of $p21^{ras}$ protein (26, 27). However, a 97-fold over-expression of $p21^{ras}$ did not increase the amount of complex detected, which would not be expected if

hsp60 participates in an essential step of p21 maturation. Nonetheless, a third limiting maturation factor may define the amount of complex we can detect. A second possibility is that hsp60 acts as a component of the effector complex through which $p21^{ras}$ releases its mitogenic and transforming signals. This model is prompted by the 2- to 3-fold increase in hsp60–p21 complex detected in mitogen-stimulated cells (20) but seems nonetheless to be unlikely, given the already characterized functions of hsp60. A remaining, attractive possibility is that hsp60 helps $p21^{ras}$ to associate with its activators or effectors. No data obtained to date bear on this possibility.

A major puzzle derives from the fact that hsp60 is reported to localize in the mitochondrial matrix (26, 27), whereas $p21^{ras}$ is reported to associate with the plasma membrane (3, 4, 30). However, it is difficult to rule out the localization of minor populations of each of these molecules to other cellular sites. Thus, we note that in *Saccharomyces cerevisiae*, a minor population of $p21^{ras}$ may be localized to organelles other than the cell membrane (34) and that its amino terminus is characterized by an amphipathic helix having positively charged residues on one side; this may represent a mitochondrial targeting sequence (35). *S. cerevisiae* and mammalian Ras protein also have reasonable mitochondrial targeting sequences at their amino termini, whereas the *Schizosaccharomyces pombe* Ras protein does not. For hsp60, there is indication that a small fraction of hsp60 is localized to the cell membrane (36, 37). Evaluation of the biological significance of the $p21^{ras}$–hsp60 interaction awaits further investigation.

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