CBA/J, and SJL mice were reverse-transcribed according to a standard protocol (*32*). Primers derived from the untranslated regions of the published Ma and Mb sequences (*10*) were used in polymerase chain reaction to amplify DNA from the first strands. This noncloned DNA was directly sequenced with the use of internal Ma or Mb primers (*32*) and fluorescent terminators. Genomic DNA derived from cosmid 5.22 (*33*) was subcloned and sequenced in the same way. For quantitation of the relative amounts of Mb1 and Mb2 transcripts, we used pairs of internal primers derived from sequences identical in both transcripts. Amplified material was divided into two portions and digested with Eae I (Mb1-specific) and Xba I (Mb2-specific), respectively. More than 90% of the material was cleaved with Xba I, less than 10% with Eae I. The amplified Ma and Mb cDNAs from B10.M were cloned. These clones were transferred to suitable expression vectors and used for all transfection experiments. Sequence analysis of spleen Mb cDNA showed that the Mβ2 amino acid sequences were identical in BALB/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$), and B10.M ($H\text{-}2^f$). In SJL ($H\text{-}2^s$) and CBA/J ($H\text{-}2^k$), amino acid 70 was Q, not H, and amino acid 96 was P, not A. In CBA/J, amino acid 105 was A, not T. Compared to the published Mα sequence, we found the following differences: B10.M, C57BL/6, BALB/c, SJL, and CBA/J all had amino acid 75 D, not G; amino acid 101 P, not S; amino acid 194 Q, not H; and amino acid 212 G, not A. C57BL/6, SJL, and CBA/J had amino acid 220 M, not I. C57BL/6 also had amino acid 9 F, not L. SJL had amino acid 7 S, not P and amino acid 89 Q, not K. Amino acid numbering is as in Fig. 4. DNA sequences are available on request. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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36. We thank D. Uranowski for secretarial assistance; J. Chambers for DNA sequencing; G. Klier for confocal microscopy; C. Suhr, M. Fukuda, and S. Pfeffer for providing antibodies; and M. Jackson, S. Schmidt, G. Aichinger, and L. Teyton for discussions and helpful comments. Supported by National Institutes of Health grant AI-26610 (P.A.P.) and by the Cancer Research Institute–F. M. Kirby Foundation (L.K.).

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Reversion of the Mouse *pink-eyed unstable* Mutation Induced by Low Doses of X-rays

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Deletions and other genome rearrangements can be caused by radiation and are associated with carcinogenesis and inheritable diseases. The *pink-eyed unstable* (p^{un}) mutation in the mouse is caused by a gene duplication and reverts to wild type by deletion of one copy. Reversion events in the mouse embryo were detected as black spots on the fur of the animals or microscopically as partially black hair in a background of colorless hair. The frequency of partially black hair was increased by x-rays at very low doses. A linear dose-response relation was found between 1 and 100 centigray.

Sources of low-level radiation are almost ubiquitous in our environment and include nuclear explosions, radiation accidents, and medical diagnostic, therapeutic, and occupational exposure. Consequently, there is much interest in the occurrence of carcinogenesis and the genetic effects associated with exposure to low-dose radiation. The Oxford childhood survey that was started in the 1950s and other studies have shown about a twofold increase in the occurrence of cancer after diagnostic intrauterine x-ray exposure at doses of approximately 2 cGy (*1*). In most cases no increased risk has been detected after exposure to doses below 10 cGy among atomic bomb survivors and individuals exposed to therapeutic irradiation (*1*). This finding may indicate a higher susceptibility of fetal tissue to radiation. Currently, no genetic end points or biological markers in animals or humans are available to detect irradiation doses close to or below 2 cGy. Only one study suggests that doses below 10 cGy may be mutagenic in human lymphoblasts (*2*). However, this result was obtained by applying 30 daily doses of 1 to 10 cGy, and no effect was found with an acute x-ray exposure of 5 cGy.

Ionizing radiation is mutagenic and carcinogenic and preferentially induces deletions rather than point mutations (*3*). Genome rearrangements such as deletions are frequently associated with tumor cells (*4*). Because of this association, a system selecting for deletions by intrachromosomal recombination has been constructed in the yeast *Saccharomyces cerevisiae* (*5*) and has been termed deletion (DEL) assay. DEL recombination can be induced with a wide variety of carcinogens, including x-rays and carcinogens that have no effect in most other short-term tests (*6*). In addition, deletion of one copy of a duplication of part of the *hprt* gene in CHO cells can be induced by x-rays and by several mutagenic carcinogens (*7*).

To determine the effect of x-ray exposure on the frequency of deletion events between two alleles of a gene duplication in mammals in vivo, we used the *pink-eyed unstable* (p^{un}) mutation in the mouse. The p^{un} mutation causes a reduction in the pigment in coat color and eye color. The p^{un} mutation is caused by a disruption of the *pink-eyed* dilute locus, which results in a DNA sequence duplication of about 70 kb in a head to tail conformation (*8*). Spontaneous reversion of p^{un} is caused by a deletion of one of the two copies of the duplicated sequence, which results in production of wild-type melanin in melanocytes. Reversion events are measured as black spots on the gray coat. The reversion frequency of p^{un} is at least three to five orders of magnitude greater than that of other recessive mutations at other coat color loci (*9*). Approximately 1.8% (*8*) to 3.8% (*10*) (5.6% in our study) of the offspring of homozygous C57BL/6J p^{un}/p^{un} mice have patches of wild-type color in their coats and are thus mosaic revertants.

Homozygous mice (C57BL/6J p^{un}/p^{un}) (*11*) were used in these experiments. An increase in reversion events would give rise to an increase in the number of animals that show dark patches. The protocol used for this test was similar to the "mouse spot test" (*12*). Matings were set up between mice homozygous for p^{un} and pregnancy was timed. Female mice were irradiated with 100 cGy of x-rays at 8.5, 9.5, and 10.5 days after conception. Dark patches on the coats of the offspring were counted, and their size and distribution were recorded. Irradiation at 8.5 days after conception caused neonatal deaths in about 40% of the offspring (Table 1). With irradiation at later stages, the viability of offspring improved, and only about 1% of neonatal deaths occurred when irradiation was done 10.5 days after conception. Less than 20% of the offspring irra-

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diated at 10.5 days after conception showed gross morphological abnormalities, whereas irradiated offspring at the earlier stages showed fewer abnormalities, similar to previous findings (*13*). Of 498 control animals, 5.6% spontaneously developed spots (Table 1). Irradiation of the female mice at 8.5 days, 9.5 days, and 10.5 days after conception increased the rate of spot development to approximately four times that in control animals ($P \ll 1 \times 10^{-6}$, chi-square distribution values).

Hair follicles contain melanocytes that secrete melanin into melanosomes, which move from the hair bulb upward into the growing hair shaft. On the 13th and 14th day after conception, the melanocyte precursor cells finish migration from the neural crest into the epidermis (*14*). The premelanocytes are first identified at day 14 and increase in number up to day 17 after conception (*15*). One hair follicle contains on the average about 13.6 melanocytes (*16*). Thus, irradiation after the 17th day after conception causing p^{un} reversion should result in parts of hairs showing black color. The entire dorsal skin of 6- to 7-day-old offspring (the age was chosen to reflect equal hair length) was divided into 12 equal sections, which were examined microscopically. Wild-type hair follicles (Fig. 1A) and hair shafts (Fig. 1D) were completely black. In comparison, follicles (Fig. 1B) and hair shafts (Fig. 1E) from p^{un} mice were almost transparent. The percentage of follicles showing black melanin were counted from six offspring (two per litter) from three litters, and the frequency of hair shafts showing black melanosomes were counted from 15 offspring (one or two per litter) from eight litters. All counted hair follicles and hair shafts from control animals showed a spontaneous frequency of black melanin streaks of about 1% (Table 2). No significant differences were seen between individual mice or between different regions of the same mouse, or between follicles and hair shafts. Six mice were irradiated with 100 cGy at day 17.5 after conception, and the offspring were killed 6 days after birth. Irradiation caused an 11- to 12-fold increase in the frequency of black melanin streaks in the follicles (Fig. 1C and Table 2) and hair shafts (Fig. 1F and Table 2) compared to control animals.

To determine whether p^{un} reversion could be induced at very low doses of x-rays, we irradiated female mice with 75, 50, 35, 9, 6, and 1 cGy. A threefold increase in the frequency of black melanosome streaks was seen at a dose of 1 cGy, and there was a linear dose-response relation between the effect of 1 cGy and 100 cGy (Table 2 and Fig. 2).

The frequency of double-strand breaks (DSBs) induced by 1 cGy of x-rays does not account for the threefold increase in the frequency of black melanosome streaks that we observed. Using a modified neutral velocity sedimentation procedure, about 70 DSBs per genome per 100 cGy of x-irradiation were reported (*17*). Thus, 1 cGy may result in about 0.7 DSBs per genome, taking into account a linear dose response for the biological activity of x-rays (*18*). The mouse genome contains about 3×10^9 base pairs, and the p^{un} duplication region is about 150 kb long. There are about 13.6 melanocytes per follicle, and reversion in any one of these may give rise to a hair with black streaks of

Table 1. Effect of x-rays on the frequency of black spots on the fur of p^{un} mice. The frequency of spots on the coats of C57BL/6J p^{un}/p^{un} mice was determined in response to x-ray exposure (*25*). Female mice were irradiated with 100 cGy of x-rays at 8.5, 9.5, and 10.5 days after conception. Dark patches in the offspring were counted, and the size and distribution of patches recorded. Offspring were examined for spots at 12 to 14 days of age, when spots are most easily visible. Two subsequent examinations were done, the last one at 4 to 5 weeks. Animal care and experimental procedures were in accordance with institutional guidelines.

Dose (Gy)	Day	No. of mice	No. of offspring	No. of live offspring	No. of spotted offspring	Frequency of spotting (%)
0				498	28	5.6
1	8.5	23	61	36	9	25
1	9.5	24	62	56	12	19
1	10.5	64	174	172	40	23
Sum of irradiation				264	61	23

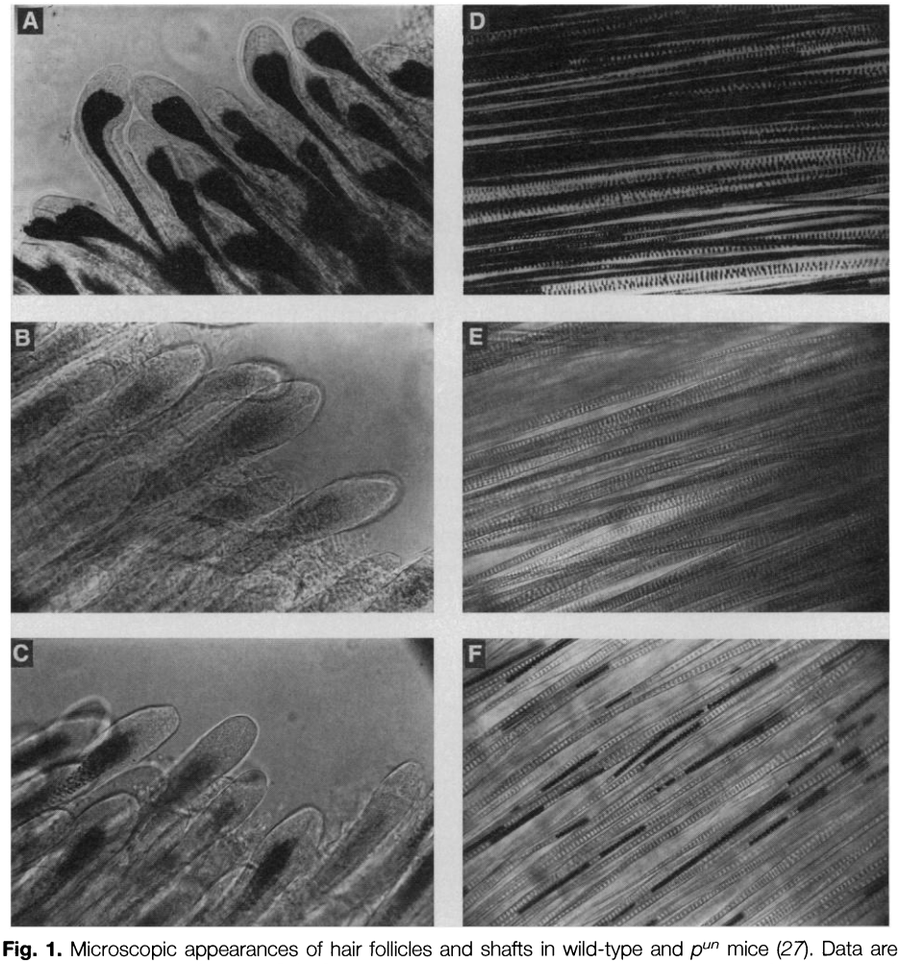

Fig. 1. Microscopic appearances of hair follicles and shafts in wild-type and p^{un} mice (*27*). Data are summarized in Table 2. (**A**) C57BL/6J wild-type follicles. Magnification, ×335. (**B**) C57BL/6J p^{un}/p^{un} follicles. (**C**) C57BL/6J p^{un}/p^{un} follicles from mice irradiated with 100 cGy of x-rays at 17.5 days after conception. (**D**) C57BL/6J wild-type hair. Magnification, ×335. (**E**) C57BL/6J p^{un}/p^{un} hair. (**F**) C57BL/6J p^{un}/p^{un} hair from mice irradiated with 100 cGy of x-rays at 17.5 days after conception.

melanosomes. This suggests a 100-fold excess of streaks of black melanosomes per 1 cGy as compared to that expected if the number of streaks of black melanosomes were the same as the number of DSBs in the p^{un} region. About 15- to 25-fold more single-strand breaks (SSBs) than DSBs are induced by x-rays (*19*). One explanation may be that all forms of DNA damage, including DSBs, SSBs, and base damage, lead to recombination. A more likely explanation may be that the induced streaks of black melanosomes at these low doses may not be caused by targeted events (DNA damage caused directly by irradiation of the DNA at the *p* locus) but may rather result from a nontargeted effect (caused by x-rays elsewhere in the cells), such as an induced state of increased recombination frequency. Such x-ray–induced states of increased spontaneous mutation frequencies have been observed (*20*) and can persist for up to 95 to 100 population doublings after irradiation. In the time between irradiation of the embryos at the 17th day after conception and the scoring of the events at the 6th day postpartum, new p^{un} revertant melanocytes could be added to the multiplying pool of revertant melanocytes during every cell generation. In addition, the shape of the dose-response curve (Fig. 2) is very shallow. There is a 12-fold effect at 100 cGy and a 3-fold effect at 1 cGy. If the events were targeted, one would expect about 1/100 the effect at 1 cGy as compared to 100 cGy for x-rays. Thus, our dose-response curve may be better explained by a nontargeted effect. For instance, different cells may have a different susceptibility to the radiation effect, and at low doses the most sensitive cells still respond.

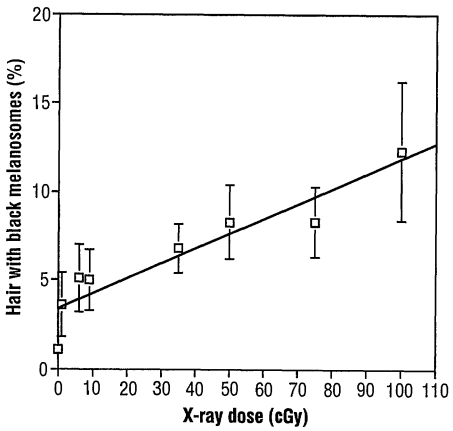

Fig. 2. Dose-response curve for x-ray–induced p^{un} reversion from 1 to 100 cGy. The error bars show the standard deviation for data obtained from different offspring. The line was calculated by the best fit for linear regression from the data in Table 2. The equation for the line was $y = 0.085x + 3.390$.

One follicle contains an average of 13.6 melanocytes (*16*); thus, the minimum frequency of induced streaks of black melanosomes is roughly 10^{-4} induced events per melanocyte per centigray at the 100-cGy dose. The rate of induced mutations per centigray of x-rays was estimated to be 2.2×10^{-7} with the "specific locus test" (*21*), and mutations have been determined to be about 2×10^{-6} per melanocyte per centigray in a cell-based version of the "mouse spot test" (*22*). Thus, the induced frequency of streaks of black melanosomes is at least 100 times greater per centigray than mutation frequencies.

The present maximum dose limit permitted annually for radiation workers is 50 mSv, which may ultimately increase the lifetime risk of cancer in these workers by more than 30% (*23*). Radiation with the effective dose of 50 mSv may cause an equivalent biological effect of 5 cGy of absorbed dose (*23*). Our data suggest that there is a linear dose-response relation for the biological effect of ionizing radiation down to very low doses in mice and that there may still be a risk at the permissible annual occupational exposure dose. Our results may indicate that deletions as genetic end points may be more sensitive to the biological activity of carcinogens than are point mutations.

Table 2. Effect of low doses of x-rays on the frequency of black melanin streaks in follicles and hair of p^{un} mice. Female mice were irradiated with 0, 1, 6, 9, 35, 50, 75, and 100 cGy of x-rays at 17.5 days after conception (*25*, *26*). The hair of all 6-day-old offspring indicated below was examined (*27*). The standard deviation applies to data obtained from different mice. The fraction of hair follicles (Fig. 1C) and of hair shafts (Fig. 1F) with reversion events was determined.

Dose (cGy)	No. of litters	No. of offspring	No. counted	No. pigmented	Percent (± SD)	Fold increase
			Hair follicles			
0	3	6	3,906	45	1.15 ± 0.97	1
100	6	12	7,512	1,053	14.0 ± 6.3	12
			Hair shafts			
0	8	15	12,218	135	1.10 ± 0.40	1
1	5	13	16,812	595	3.6 ± 1.8	3.3
6	7	11	14,807	797	5.1 ± 1.9	4.6
9	5	13	13,602	669	5.0 ± 1.7	4.5
35	4	7	8,699	588	6.8 ± 1.4	6.2
50	6	14	14,779	1,236	8.3 ± 2.1	7.6
75	5	13	15,927	1,334	8.3 ± 2.0	7.6
100	6	12	6,609	802	12.3 ± 3.9	11.2

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25. The protocol used for this test was similar to the "mouse spot test" (*12*). Matings were set up between mice homozygous for p^{un}, and pregnancy was timed from the discovery of a vaginal plug. First and second litters were used and gave the same results. Sperm entry into the egg was assumed to have occurred in the early morning hours of the day on which the plug was found, and the noon time of this day was defined as 0.5 days after conception. According to the "mouse spot test," the best time of treatment lies between the 9th and the 11th day after conception (*24*). However, for application of the "mouse spot test" at the single-hair level, two peaks

of sensitivity, at the 12.5th and the 17.5th day after conception, were observed (*22*). For irradiation, a Westinghouse 150 Industrial X-ray Machine was used (130 kilovolt peak–x-rays, delivered by a self-rectifying tube, inherent filtration 1.65-mm aluminum). A current of 8 mA was used to obtain an intensity at 40 cm of 24 ± 2 rad/min. The mice were exposed in individual sterile polypropylene-polyethylene containers resting on a 24-cm-diameter steel turntable. The mice were rotated to ensure a more accurate average value of the irradiated field. The delivered dose was measured for each irradiation with a Victoreen C-r 570 meter.

26. These doses were read from the radiometer that was put into the irradiation chamber next to the mouse container.
27. The experiments with single hairs were done similarly to experiments described for a single cell–based assay of the "mouse spot test" (*16*). The six unirradiated offspring were from three independent litters. Six mice were irradiated with 100 cGy at day 17.5 after conception, and 12 offspring were used. The skin of the mice was prepared as described by Searle and Stephenson (*16*). The animals were killed by decapitation 6 or 7 days after birth. Blood was allowed to drain away, and each mouse was dissected to remove the skin. The skins were then treated with 10% formalin for at least 24 hours, washed in distilled water, transferred to 70% ethanol for 24 hours, transferred to 90% ethanol for 24 hours, and incubated in two changes of absolute ethanol for 12 hours each. The skins were then treated with a 1:1 benzyl:ethyl alcohol mixture for 12 hours and with pure benzyl alcohol for 12 hours. Any adherent muscle tissue was removed with blunt-end forceps to leave the skin as thin as possible. The skin was divided into 12 dorsolateral segments, and each segment was mounted in Canada balsam with the dorsal surface uppermost on a microscope slide under a cover slip. The slides were then left to dry on a hot plate at 40°C. Each slide was examined in a bright field under 100-fold magnification and the follicles and hair counted.
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Association of Insulin Receptor Substrate–1 with Integrins

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Insulin stimulation was found to promote association of the $\alpha_v\beta_3$ integrin (a vitronectin receptor) with insulin receptor substrate–1 (IRS-1), an intracellular protein that mediates insulin signaling by binding other signaling molecules, including growth factor receptor–bound protein 2 (Grb2) and phosphatidylinositol-3′ kinase. Insulin-treated cells expressing the $\alpha_v\beta_3$ integrin showed 2.5 times more DNA synthesis when plated on vitronectin than on other substrates, whereas cells expressing another vitronectin receptor, $\alpha_v\beta_5$, did not show this difference. The association between integrin and IRS-1 may be a mechanism for the synergistic action of growth factor and extracellular matrix receptors.

Adhesion of cells to extracellular matrix (ECM) is mainly mediated by the integrin family of cell surface receptors (*1*) and is a prerequisite for cell proliferation and survival (*2*). The pathways for integrin signaling and growth factor signaling are thought to be mechanistically linked because cell adhesion to ECM is required for cells to respond to certain growth factors (*3*) and integrin-mediated cell adhesion and motility can be modulated by growth factors (*4*). Furthermore, growth factor treatment can disrupt focal adhesions, the presumed sites of integrin-mediated signaling (*5*).

To investigate whether integrins associate with molecules involved in growth factor signaling, we studied Rat-1 fibroblasts that had been stably transfected with DNA encoding the human insulin receptor (HIRcB cells) (*6*). We immunoprecipitated integrins from these cells and searched for coprecipitated tyrosine-phosphorylated proteins by immunoblotting with antibody to phosphotyrosine (anti-pTyr). Two polyclonal antibodies against the $\alpha_v\beta_3$ integrin and a polyclonal antibody against the platelet $\alpha_{IIb}\beta_3$ integrin coprecipitated a 185-kD phosphorylated protein (p185) from insulin-stimulated but not quiescent HIRcB cells (Fig. 1A, lanes 1, 3, 4, and 5). Two other polyclonal antibodies to $\alpha_v\beta_3$ did not coprecipitate p185, possibly because they disrupt the integrin-p185 association (*7*). No coprecipitation of p185 occurred with polyclonal antibodies against the $\alpha_5\beta_1$ integrin (a fibronectin receptor) (Fig. 1A, lane 6) (*8*) or with antibodies against unrelated proteins (Fig. 1A, lane 7). Similar results were obtained with human HepG2 hepatoma cells (*9*). When anti-$\alpha_v\beta_3$ immunocomplexes were dissociated in SDS and reprecipitated with the same antibodies, p185 was not detectable in the reprecipitated receptor complex (*10*), suggesting that antibodies to integrins do not directly cross-react with p185 under these conditions (Fig. 1A, lane 8).

Isolation of HIRcB cell integrins on an affinity column confirmed the association of p185 with integrins. The phosphorylated p185 copurified with detergent extracts from insulin-treated cells on GRGDSPK-Sepharose, a known ligand for $\alpha_v\beta_3$ and other integrins (*11*), but not on Sepharose linked to a control peptide, GRGESPK (Fig. 1B).

Insulin receptor substrate–1 (IRS-1) is the major target protein phosphorylated on tyrosine by ligand-activated insulin receptor and has an apparent molecular mass of 185 kD on SDS-polyacrylamide gels (*12*). Immunoblot analysis with an antiserum against the NH_2-terminus of IRS-1 (*13*) demonstrated the presence of a reactive band in anti-$\alpha_v\beta_3$ immunocomplexes from insulin-stimulated but not quiescent HIRcB cells (Fig. 1C). Similar results were obtained with two other polyclonal antibodies to IRS-1, one raised against the COOH-terminus of IRS-1 and the other against recombinant IRS-1 produced in insect cells (*14*). The antiserum against the NH_2-terminus of IRS-1, however, was used throughout this study, as was anti-$\alpha_v\beta_3$ 237, unless otherwise indicated. Dissociation of the anti-$\alpha_v\beta_3$ immunocomplexes from insulin-treated cells and subsequent reprecipitation with anti–IRS-1 yielded a band corresponding to IRS-1 (Fig. 1D). The fact that the bands produced by anti-$\alpha_v\beta_3$ and anti–IRS-1 (Fig. 1D, lanes 1 and 2) were of about equal intensity, and the failure of the phosphotyrosine antibody to immunoprecipitate any phosphotyrosine-containing proteins from the dissociated anti-$\alpha_v\beta_3$ complexes after they had been depleted of IRS-1 (Fig. 1D, lane 3), showed that IRS-1 is a major portion of p185 associated with the vitronectin receptor. About 5 to 8% of IRS-1 associated with the vitronectin receptor, and maximal association was reached after 3 min of insulin stimulation (*15*). The insulin receptor was not detectable in the integrin immunocomplex (*14*).

Tyrosine-phosphorylated IRS-1 links insulin receptor activation to downstream intracellular signaling pathways through its association with proteins containing the Src homology 2 (SH2) domain. These proteins include the Ras guanine nucleotide–releasing complex Grb2-Sos, phosphatidylinositol-3′ kinase (PI-3 kinase), the phosphotyrosine phosphatase Syp, and the adaptor protein Nck (*12*). We found that at least two of these proteins, Grb2 and PI-3 kinase, were precipitated by anti-$\alpha_v\beta_3$ from the insulin-stimulated HIRcB cells (Fig. 2). Whether other IRS-1–binding proteins are also associated with integrins after insulin stimulation remains to be tested.

To investigate the physiological effects of the integrin–IRS-1 association, we studied FG human pancreatic carcinoma cells which do not express $\alpha_v\beta_3$, but rather use

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